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Sheep urine patch nitrous oxide emissions: measurement and mitigation

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Sheep urine patch nitrous oxide emissions: Measurement and mitigation

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Thesis Summary

Demand for livestock products are expected to rise due to increasing global population, urbanisation and affluence. Sustainably managing livestock excreta will be central to achieving an expansion in production whilst minimising environmental damage. The deposition of excreta to soil by livestock accounts for ca. 21% of the UK agricultural N_2O emissions. Accurate quantification of N_2O and assessments of the efficacy of mitigation technologies are key research areas for progressing toward enhanced sustainability and productivity in grazed grasslands. The overall thesis aims are to enhance understanding of N cycling and losses in sheep urine patches, as 'hot-spots' and 'hot-moments' for rapid nutrient cycling. Objectives were (i) to determine how sheep urine patch and environmental parameters influence N_2O emissions, (ii) to determine the optimal way to accurately measure N_2O emissions from sheep urine patches via the static chamber technique, and (iii) to assess the efficacy of synthetic nitrification inhibitors as a mitigation strategy for urine patch N_2O emissions. Sheep-grazed grasslands were selected for study, based on the lack of current available evidence for these agroecosystems. N_2O emissions were monitored from sheep urine-influenced soil in small incubation vessels, or by the static chamber technique in the field (manual and automated campaigns). The use of ^{14}C-labelled inhibitors were also employed in laboratory studies, to trace the fate of nitrification inhibitors in the plant-soil-microbe system and provide a better understanding of the factors that affect the efficacy of inhibitors to reduce N_2O emissions. Urine patch size and N concentration were found to be important parameters influencing emissions of N_2O from sheep urine patches. Emissions of N_2O were generally lower than the IPCC default of 1% of the N applied in sheep excreta, where peaks in emissions occurred alongside rainfall events. Total extractable N, oxidation reduction potential and soil water-filled pore space were determined to be key drivers of N_2O emissions from sheep urine under controlled conditions. The urine patch diffusional area was shown to be important for accurate quantification of N_2O emissions when using the chamber technique; the importance of daily sampling of emissions, an assessment of the diurnal nature of N_2O emissions and having a high number of replicate chambers to adequately represent the large spatial variability in N_2O emissions was also confirmed. The nitrification inhibitors DCD and DMPP had contrasting behaviours in differing soil types. DCD had a greater sorption in comparison to DMPP and microbial uptake and degradation were concluded to be important parameters influencing their effective period in the soil. A graminaceous plant was shown to be able to acquire DCD intact through its roots and translocate the compound to shoots which raises concerns about contamination of food products. A liquid application of DMPP was not effective in reducing cumulative N_2O emissions from sheep urine patches in the field. The efficacy of nitrification inhibitors to reduce N_2O appears to vary widely, nevertheless they are a mitigation strategy that could be implemented in the short term. Achieving enhanced sustainability and productivity in grazed grasslands is a complex problem, requiring an interdisciplinary approach and the involvement of policy-makers and farmers to resolve. There are several mitigation strategies available or being developed, and some which require more research before being practicable. Advances in technologies to measure and mitigate N_2O emissions will greatly enhance our knowledge of N cycling and losses, and the potential to alleviate such losses from the urine patch environment in the near future.

Thesis Contents

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Abbreviations

AMO - Ammonia monooxygenase

ANOVA - Analysis of variance

C - Carbon

Ca - Calcium

CEC - Cation exchange capacity

CH_4 - Methane

CO_2 - Carbon dioxide

DCD - Dicyandiamide

DEFRA – Department of Environment Food and Rural Affairs

DMPP - 3,4-Dimethylpyrazole phosphate

DMSO - Dimethylsulfoxide

DNRA - Dissimilatory nitrate reduction to ammonia

DOC - Dissolved organic carbon

DON - Dissolved organic nitrogen

DOP – Dissolved organic phosphorus

DW - Dry weight

EC - Electrical conductivity

ECD - Electron capture detector

EF - Emission factor

FAO - Food and Agriculture Organization

FID - Flame ionization detector

FW - Fresh weight

GHG - Greenhouse gas

GC - Gas chromatograph

GPS - Global positioning system

HAO - Hydroxylamine oxidoreductase

HPLC - High performance liquid chromatography

IPCC - Intergovernmental Panel on Climate Change

K - Potassium

LSD - Least squares difference

N_2 - Nitrogen molecule

Na - Sodium

NAR - Nitrate reductase

NH_3 - Ammonia

NH_4^+ - Ammonium

NI - Nitrification inhibitors

NIR - Nitrite reductase

NO_2^- - Nitrite

NO_3^- - Nitrate

N_2O - Nitrous oxide

NO - Nitric oxide

NO_2 - Nitrogen dioxide

NO_x - Nitric oxide and/or nitrogen dioxide

NOR - Nitric oxide reductase

NOS - Nitrous oxide reductase

NUE - Nitrogen use efficiency

O_2 - Oxygen

OCT - Organic cation transporter

ORP - Oxidation reduction potential

P - Phosphorus

PLFA - Phospholipid fatty acid

PO_4^{3-} - Phosphate

SEM – Standard error of the mean

UK - United Kingdom

UV - Ultraviolet

WFPS – Water-filled pore space

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Chapter 1

Introduction

Sheep urine patch nitrous oxide emissions: Measurement and mitigation

1.1 Introduction to the challenges facing the livestock industry

Global demand for livestock products are set to increase in the future, due to projected increases in global population to 9 billion by 2050, increased income and urbanization (Smith et al., 2010; Thornton, 2010). Yet, the livestock sector is already estimated to contribute 14.5% of the global anthropogenic greenhouse gas (GHG) emissions (Gerber et al., 2013), in the form of carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O). Emissions of these three GHG's reflect losses of energy, organic matter and nitrogen (N) from agricultural systems (Eckard et al., 2010; Gerber et al., 2013). The major challenge facing the livestock sector is to meet increased demand efficiently and sustainably, whilst curbing its associated environmental impacts. In recent years, the potential for mitigation of non-CO_2 GHG's have been increasingly recognised (Reay et al., 2012).

1.2 Thesis background and rationale

The research presented herein, is situated within the biogeochemical N cycle of grazed pastures (Figure 1), and specifically pertains to urine patches deposited by sheep. These sites are recognised as 'hot-spots' and 'hot-moments' for N_2O emissions and other N loss processes (see Figure 1; van Groenigen et al., 2005; Cowan et al., 2015), undermining the efficiency of grazed grasslands. For a detailed analysis of N cycling within urine patches, readers are referred to Chapter 2 (a review of the literature).

A biogeochemical 'hot-spot' is defined as patches with disproportiantely high reaction rates in comparison to the surrounding area (McClain et al., 2003) e.g. a small volume of soil with accelerated microbial processing rates compared to surrounding soil conditions. The concept does not solely apply to one biogeochemical process (Hagedorn and Bellamy, 2011), however, the large spatial variability in GHG emissions from soil are often attributed to hot-spots. Such GHG emission hot-spots require a supply of reactants and favourable conditions

for GHG production (McClain et al., 2003; Hagedorn and Bellamy, 2011; Kuzyakov and Blagodatskaya, 2015). A 'hot-moment' is defined as time periods with higher microbial processing rates in comparison to intervening periods (McClain et al., 2003). Hot-spots and hot-moments can occur separately, or at the same time. The urine patch supplies additions of reactive N, C and moisture to soil, removing many of the limitations to microbial activity and can stimulate the major N_2O producing pathways of nitrification and denitrification (Firestone and Davidson, 1989; Wrage et al., 2001; Bateman and Baggs, 2005). Thus, urine patches in grasslands can be described as hot-spots and hot-moments for N_2O emissions.

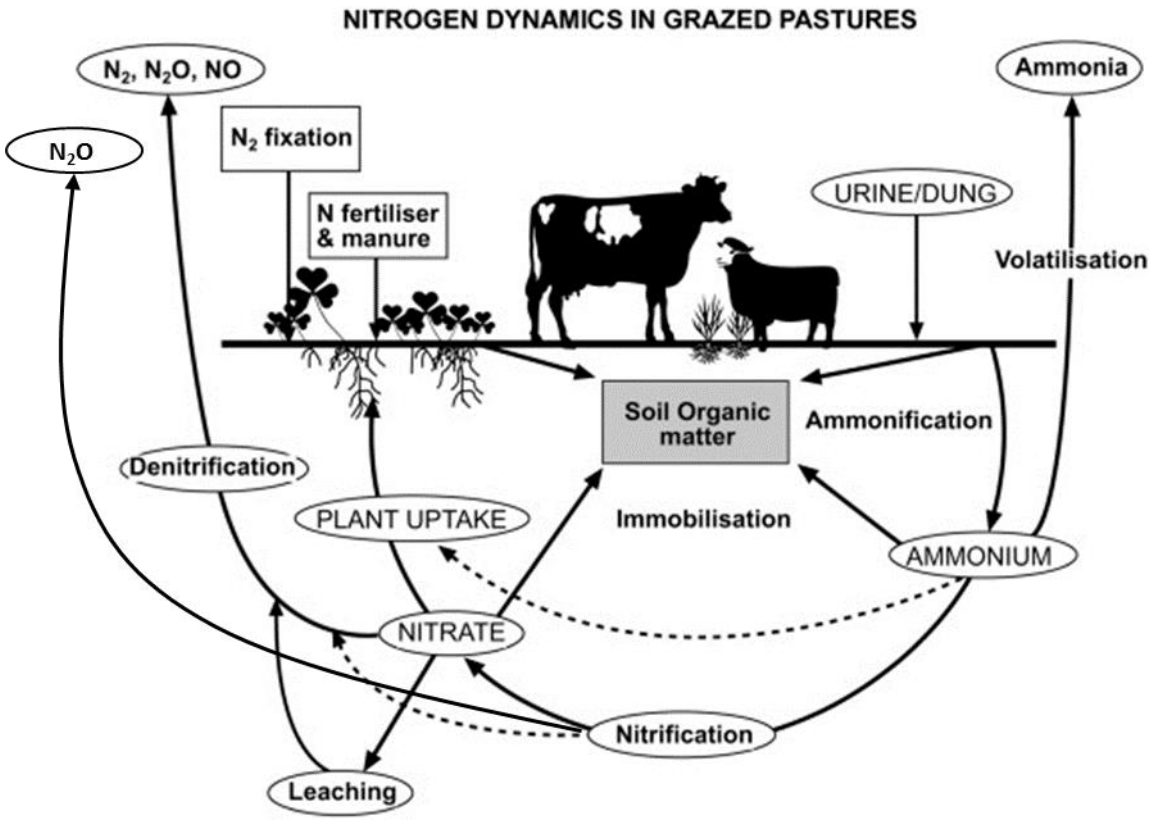

Figure 1 The nitrogen cycle of grazed pastures, adapted from Saggar et al. (2013).

Due to the myriad of edaphic, environmental and management practices which can influence N cycling in the animal-plant-soil-atmosphere system, there are large uncertainties associated with broad-scale estimates of N_2O emissions from grazed grasslands (Hagedorn and

Bellamy, 2011; Butterbach-bahl et al., 2013). Nevertheless, recent estimates of the N_2O emissions arising from grazing returns to grasslands are ca. 40% of the global anthropogenic N_2O emissions arising from animal production systems (Oenema et al., 2005) and ca. 21% of the emissions of N_2O from UK agriculture (UNFCC, 2015). This represents one of the three largest sources of N_2O from agriculture, alongside leaching and fertiliser applications, representing a potentially large emission reduction potential from the livestock sector.

This thesis broadly focuses on the measurement and mitigation of N_2O from grassland soils, at the scale of the urine patch. Both accurate quantification of N_2O emissions and assessments of suitable mitigation technologies are key research areas for making progress toward combatting the current challenges identified for the livestock sector, to reduce its GHG emissions. Sheep-grazed grasslands were selected for study, based on a need for specific data pertaining to such systems. This contrasts with urine patches in cattle based systems for which a large amount of scientific literature already exists.

For N_2O emission measurements undertaken in this thesis, the use of gas chromatography was employed, where headspace gas samples were taken from small incubation vessels in laboratory studies, and from static-chambers in field studies. The standard chamber technique (Hutchinson and Mosier, 1980) is a widely employed technique for measuring N_2O fluxes from field soils. Although several new advances in analytical technology for the measurement of N_2O from soils are being made (this will be discussed in Chapter 2), the chamber technique is still likely to be used for the foreseeable future, due to its low cost. Therefore, methodologies for the accurate quantification of N_2O emissions from urine patches, using this strategy, are warranted. An automated static chamber based system was also used, allowing measurements of N_2O emissions from urine patches at a high temporal resolution (8 flux measurement per 24 h). This allowed an assessment of different sampling strategies based on varying the frequency of measurements.

There are a wide range of techniques which could be employed to mitigate N_2O emissions from ruminant animals and urine patches, and this thesis focuses on only one potential strategy - the use of nitrification inhibitors. These compounds can be potentially utilised by application to the soil (Di and Cameron, 2003), by incorporating into livestock diet (Ledgard et al., 2008) or via addition to slurry and manure (Dittert et al., 2001; Hatch et al., 2005). The mechanisms and use of this technology in ruminant urine-influenced soil is reviewed in Chapter 2. Briefly, this mitigation technology was selected for research due to its potential to provide multiple benefits including improvements in nitrogen use efficiency, reductions in nitrate leaching and losses of gaseous N (Di and Cameron, 2002; Di and Cameron, 2007; Ruser and Schulz, 2015). Such techniques which have multiple benefits are likely to be important tools in any future mitigation strategies. However, the efficacy of nitrification inhibitors to provide such benefits varies widely under different edaphic and climatic conditions (Barth et al., 2001; Kelliher et al., 2008; Menéndez et al., 2012; Abalos et al., 2014). Further research is also required for determining their practicality, particularly in sheep urine-influenced soils. This thesis explores some of the factors influencing nitrification inhibitor efficacy, and focuses on two of the most commonly used nitrification inhibitors, dicyandiamide (DCD) and 3,4-dimethylpyrazole phosphate (DMPP) (Liu et al., 2013). Here, the use of ^{14}C-labelled nitrification inhibitors was employed in laboratory studies, to trace the fate of these inhibitors in the soil-microbe-plant system.

1.3 Thesis aims and objectives

This section details the aims and objectives of the thesis, followed by a brief description of the relevant chapters which include experimental work pertaining to each objective. A list of the experimental chapter titles can be found in section 1.8; for individual experimental chapter hypotheses, please refer to the prepared manuscripts. Discussion points arising from

the overall objectives are included as bullet points in section 1.9. The purpose of these are to relate the insights gained from the current research, with recommendations for the direction of future research. This forms the basic structure of the final discussion section of the thesis (Chapter 8).

1.3.1 Thesis aims

The overall aims of the thesis are to enhance understanding of N cycling and losses in urine patches, as hot-spots and hot-moments for rapid nutrient cycling; to determine how to accurately measure N_2O emissions from sheep urine patches via the chamber technique, and to assess the efficacy of synthetic nitrification inhibitors as a strategy to reduce N_2O emissions from sheep urine patches.

1.3.2 Objective 1

Determine how a) sheep urine patch parameters and b) environmental parameters influence N_2O emissions from grassland soils.

a) Sheep urine patch parameters

Urine patches vary widely in terms of N content, composition, patch size, volume and frequency (Betteridge et al., 2010). In Chapter 3, the horizontal diffusion of solutes out of the urine patch is assessed, investigating how this influences N_2O emissions. In Chapter 4, the effects of urine patch size, N content and volume on N_2O emissions are investigated within the typical ranges reported for sheep. In Chapter 7, average sheep urine volumes for Welsh Mountain ewes are determined and N_2O emissions are quantified under field conditions.

b) Environmental parameters

Other than reactive N inputs, the main drivers of N_2O emission from soil include moisture status (related to the soil oxygen status), temperature and the availability of labile C

(Smith, 2010; Butterbach-Bahl et al., 2013). The influence of soil water-filled pore space on emissions of N_2O from urine-influenced soil is investigated under controlled conditions in Chapter 3. In Chapters 4 and 7, urine N is applied under different seasons including spring and late summer, respectively. This allows some assessment of N_2O emissions under differing weather patterns, due to seasonality. The effect of spatial heterogeneity in soil conditions on N_2O emissions from urine patches is also discussed in Chapter 7.

1.3.3 Objective 2

Determine how to accurately monitor N_2O emissions from sheep urine patches, using the closed chamber technique.

In Chapter 3, the importance of including the urine patch diffusional area for monitoring N_2O emissions is assessed, which has implications for urine application methods within chambers, under experimental conditions. The high frequency automated chamber system was used in Chapters 4 and 7. In Chapter 4, the data are used to determine how different sampling strategies can influence the accuracy of N_2O emission measurements from sheep urine patches. In Chapter 7, the diurnal nature of GHG fluxes are assessed, and insights gained are considered in the context of reduced frequency chamber measurements. Additionally, Chapter 7 considers how automated chamber based systems can be optimised for capturing both spatial and temporal variability in N_2O emissions.

1.3.4 Objective 3

Investigate the factors which influence nitrification inhibitor efficacy in ruminant urine-influenced soils and assess their potential as an N_2O mitigation strategy for sheep-grazed grasslands.

Factors which influence the efficacy of nitrification inhibitors in soils are assessed in Chapters 5, 6 and 7. In Chapter 5, a comparative study was conducted for DCD and DMPP to determine their vertical mobility, and potential dis-location from urine derived ammonium, in a range of soils contrasting in texture and organic matter content. Chapter 6 assesses the magnitude of plant acquisition of DCD, both as a DCD depletion pathway and as a potential vector for contamination of food products. In Chapter 7, the direct measurement of the efficacy of DMPP in reducing N_2O emissions from real sheep-derived urine patches, under summer conditions are investigated, varying the time-since DMPP application.

1.4 Experimental chapter information

The experimental chapters of the current thesis have been prepared in the style of journal article manuscripts. The title page of each experimental chapter contains details of the authors, author contributions to the manuscript, the current progress of each manuscript (e.g. published / accepted / submitted / not yet submitted) and any additional information, such as whether the work has / will be presented at a conference. The thesis consists of five experimental chapters, located in Chapters 3-7 of the current document. For continuity and clarity, the experimental chapters will be referred to as they appear in this thesis. The titles of each experimental chapters are as follows:

Chapter 3: The urine patch diffusional area: an important N_2O source?

Chapter 4: Disentangling the effect of sheep urine patch size and N loading rate on cumulative N_2O emissions.

Chapter 5: The mobility of nitrification inhibitors under simulated ruminant urine deposition and rainfall: a comparison between DCD and DMPP.

Chapter 6: Plant acquisition and metabolism of the synthetic nitrification inhibitor dicyandiamide and naturally-occurring guanidine from agricultural soils.

Chapter 7: Effect of time-since DMPP application on mitigating N_2O emissions from sheep urine patches.

1.5 Discussion points arising from objectives

The results and conclusions of the objectives of the thesis will be discussed in Chapter 8, where the following questions will form the basis of the structure of the discussion:

- What implications do variations in urine patch and environmental parameters have on the upscaling of N_2O emissions from grazed grasslands?
- How should the chamber technique be deployed, to monitor N_2O emissions from urine patches?
- Are nitrification inhibitors a suitable strategy for reducing N_2O emissions from grazed grasslands?
- What alternative strategies are there for the mitigation of N_2O emissions from urine patches?
- How should future research be prioritised, in order to achieve enhanced productivity and sustainability in grazed grasslands?

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Chapter 2

Literature Review

2.1 Introduction

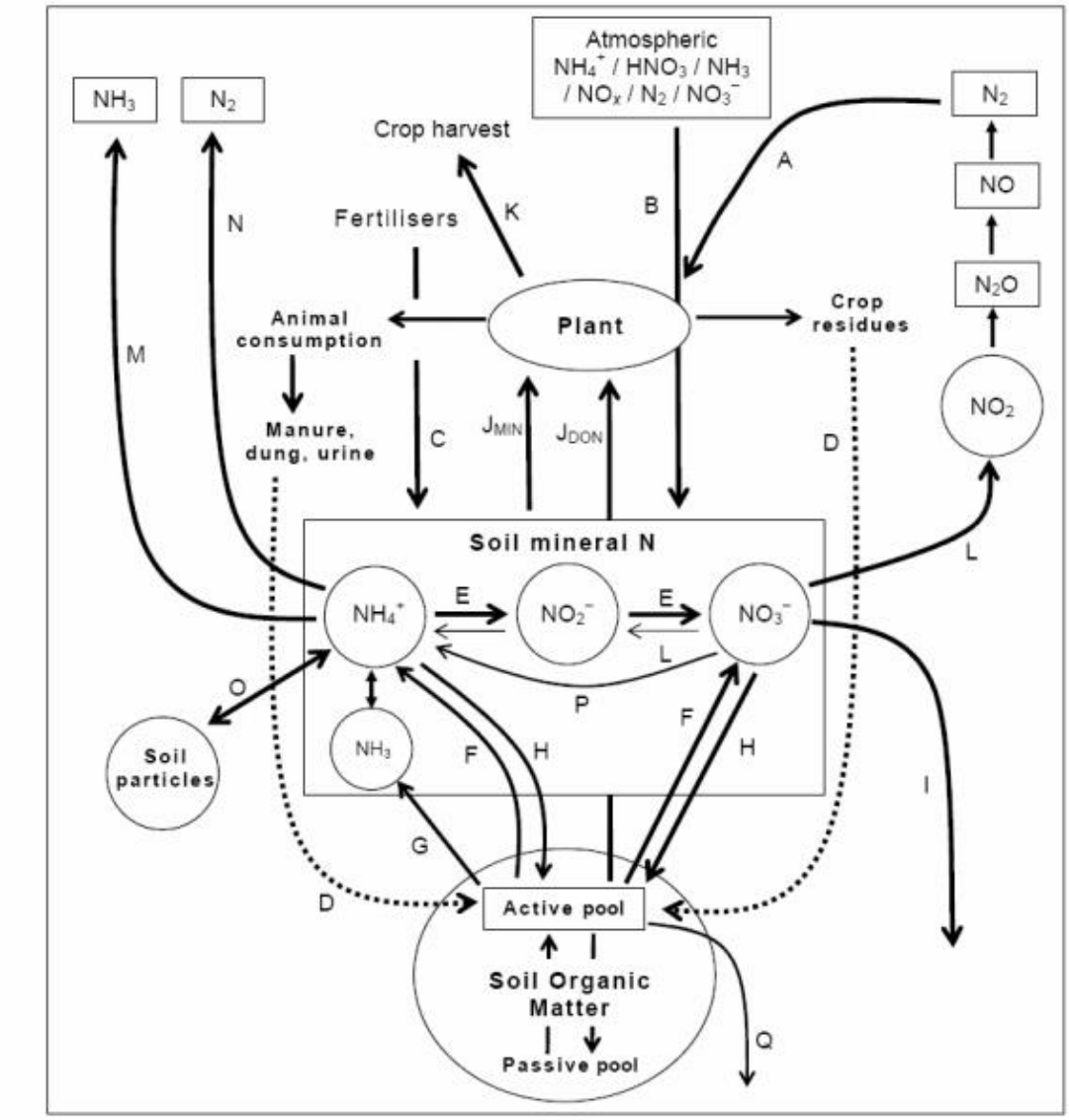
Over the past few decades, there has been a large worldwide increase in the number of domestic animals, due to increasing consumption of meat protein *per capita* (Oenema et al., 2005). It is projected that the global population will reach 9 billion by 2050 (Smith, 2010), and the demand for meat and milk-based products is projected to rise by 68% and 57%, respectively, by 2030 compared to the year 2000 (FAO, 2006). Sustainably managing livestock excreta will be central to achieving an expansion in production with minimal environmental damage (Davidson, 2009; Chadwick et al., 2015). This literature review focuses on the urine patch of grazing animals as sites of accelerated nitrogen (N) cycling processes and as discrete sources of N losses from grazed pastures. The review will demonstrate that these sites can contribute to environmental, economic and human health issues as the reactive N cascades through different chemical forms in the biosphere, hydrosphere and atmosphere (Bolan et al., 2004; Sutton et al., 2011). The literature relating to urine patch research is largely dominated by cattle-based systems, therefore, this review aims to assess current understanding of N cycling and losses with specific reference to sheep urine patches, although comparisons to cattle based systems are made where necessary.

This review focusses initially on the reactive N cascade (the ability of reactive N to transition through several chemical forms, causing a series of negative effects on ecosystems and human health; Galloway et al. (2010)), as a necessary background to understanding the complexity of N cycling in the environment and from livestock production systems. Current knowledge of sheep urine composition and urine patch characteristics are then reviewed, followed by terrestrial N transformations and drivers of N losses. Techniques to measure nitrous oxide (N_2O) emissions from urine patches are assessed and the use of nitrification inhibitors as a means to reduce N losses from the grazed pasture system are also considered in detail.

2.2 The reactive N cascade in the context of livestock production

Nitrogen limits primary productivity in most ecosystems (LaBauer and Treseder, 2008), despite being the most abundant element in the atmosphere (Pelletier and Tyedmers, 2010). This is because N mostly resides as the non-reactive N_2 molecule, and the forces required to break the strong triple bond of the N_2 molecule renders it unusable to most living organisms (Galloway et al., 2003). Reactive N has been defined by Galloway et al. (2003) as all inorganic reduced forms of N (NH_3, NH_4^+), inorganic oxidized forms of N (NO_x, HNO_3, N_2O, NO_2^-, NO_3^-) and organic forms of N (e.g. urea, amino acids, proteins, nucleic acids, amines), which are essential components of the building blocks of life (Galloway and Cowling, 2002). Prior to human intervention, the amounts of reactive N in circulation was limited, due to biological N fixation by specialised microorganisms (e.g. free-living bacteria, blue-green algae and N-fixing bacteria associated with the roots of leguminous plants) being the dominant source of reactive N creation (Galloway and Cowling, 2002). In the nineteenth century the development of the Haber-Bosch process, an industrial process capable of producing reactive N for fertilizer, was fundamental for being able to produce enough food for a growing population (Galloway et al., 2010). However the concurrent rise in the use of fertilizers, cultivation of legumes and fossil fuel consumption, have contributed to a dramatic increase in reactive N creation (Galloway et al., 2004; Bouwman et al., 2009).

Livestock systems fit into the global N cycle (see Fig. 2.1) by converting reactive N stored in plant resources, which are of no immediate other use, into human-edible protein (Steinfeld and Gerber, 2010; Dijkstra et al., 2013). Grasslands cover 20-40% of the land surface of earth, depending on the definition used (Panunzi, 2008), and fodder produced to feed animals accounts for ca. 30% of all arable land (Steinfeld et al., 2006; Bouwman et al., 2013). Both plants and animals associated with livestock production are highly inefficient at incorporating applied N into their tissues. Grazing animals excrete a large proportion (70-95%) of the N they



A	Symbiotic and asymbiotic N fixation		dissolved organic N (J_{DON})
B	Wet and dry depositions	K	Removal of N from soil by harvest
C	Mineral fertiliser inputs	L	Denitrification (biotic and abiotic)
D	Organic matter inputs	M	Volatilisation
E	Nitrification	N	Anammox (anaerobic ammonium oxidation): $NH_4^+ + NO_2^- \rightarrow N_2 + 2\ H_2O$
F	Mineralisation		
G	Ammonification	O	Ammonium fixation and desorption
H	Immobilisation	P	Dissimilatory nitrate reduction to ammonium (DNRA)
I	Leaching of mineral N		
J	Crop uptake of mineral (J_{MIN}) and	Q	Leaching of dissolved organic N

Figure 2.1 Schematic of the N cycle. Source: Stark and Richards (2008).

consume (Oenema et al., 2005), mainly because the amount of N required for the optimal growth of vegetative material exceeds the amount of N that grazing animals require for protein synthesis (Ussiri and Lal, 2012). As a result, labile nutrients from the consumed, digested and excreted plant material are returned to soil, at a greater rate than the comparative degradation of plant litter (Harrison and Bardgett, 2008). The urine fraction is considered more labile than the faeces, where up to 60% (Haynes and Williams, 1993) of consumed N is excreted. In addition, there is estimated to be more N stored in managed manure produced by livestock, than there is in global N fertilizer use (Bouwman et al., 2009; Bouwman et al., 2013), highlighting the importance of livestock systems as contributors to elevated levels of reactive N in the environment. In the UK, the N input from excreta N and inorganic N are similar e.g. input from manure: 323 kt N $year^{-1}$, N excretion on pasture range and paddock: 613 kt N $year^{-1}$ and N inout from inorganic fertilizers applied to ropland and grassland: 1150 kt N $year^{-1}$ (Laura Cárdenas, personal communication, 2016).

The pathways of N removal processes (Fig. 2.1) from agricultural systems include N offtake in the form of crops/livestock products, volatilisation of NH_3, losses of N to water by leaching of NO_3^- and lateral flows (e.g. run off and erosion), denitrification and release of gaseous N compounds (NO, NO_2, N_2O, N_2) and the anaerobic oxidation of NH_4^+ to N_2 (anammox). Some of these N removal processes are environmentally benign e.g. conversion of reactive N to N_2, yet others have far-reaching consequences for human health and the environment. Atmospheric N species can be re-deposited to land through wet (e.g. by dissolving in water droplets) or dry deposition (e.g. deposition of particulate matter). Atmospheric NH_3 is present in the atmosphere in gaseous form, but can readily dissolve in atmospheric water or react with acidic gases or particles to form hygroscopic salts, atmospheric NH_4^+ is generally in particulate form (Mosier, 2001). Atmospheric NO_3^- occurs as particulates

(Orel and Seinfeld, 1977), whereas NO_x, N_2 and HNO_3 exist in gaseous form, and the latter can dissolve in water droplets (Hanke et al., 2003).

Gaseous losses of N which are of environmental concern include N_2O and nitric oxides (NO and NO_2; collectively known as NO_x). N_2O emissions are one of the major environmental implications associated with livestock production (Ambus et al., 2007; Kool et al., 2006), and the urine patches of grazing animals are recognised hot spots for N_2O emissions from grazed grasslands. In literature reviews conducted by van Groenigen (2005) and Selbie et al. (2015), the average fraction of applied ruminant urine N emitted as N_2O was found to be 1.7% (n = 31) and 2.1% (n = 40). These are similar to the default IPCC emission factor used for cattle excreta (2% of applied N) in Tier 1 inventories, and likely reflects the greater proportion of studies on cattle urine patches compared to sheep urine patches. The default IPCC emission factor for sheep urine is 1% of the applied urinary N, due to lower urine volumes and lower compaction in sheep grazed grassland (IPCC, 2006). N_2O is a potent greenhouse gas (Orwin et al., 2010), with an atmospheric lifetime of over 100 years and a global warming potential ca. 300 times that of carbon dioxide (CO_2; Wrage et al., 2001). The increase in N_2O since the industrial period contributes an additional radiative forcing of 0.16 ± 0.02 W m^{-2} and is primarily anthropogenic in origin (IPCC, 2007). Of the global total N_2O emissions from animal manure in 2000, 41% was attributed to grazing, where the highest emissions on a per animal basis were from non-dairy cattle, followed by sheep (Oenema et al., 2005). Indirect emissions of N_2O can also occur when N compounds are lost via volatilisation (followed by N deposition), runoff and leaching. The lost N can subsequently be transformed to N_2O in a different location (e.g. freshwater sediments, drainage ditches; Mosier et al., 1998; Oenema et al., 2005), as part of the reactive N cascade.

In addition to the global warming potential of N_2O residing in the troposphere, stratospheric depletion of ozone is another side effect. N_2O can react with atomic oxygen to

form nitric oxide (NO) in the stratosphere, inducing ozone destruction (Crutzen, 1970; Davidson et al, 2000; Wrage et al., 2001). This gas is currently the dominant ozone-depleting substance in the atmosphere, surpassing chlorofluorocarbons, which have seen a successful reduction in emissions following the Montreal Protocol (Ravishankara et al., 2009). A depletion of ozone in the stratosphere is associated with increased risk of skin cancer and damage to plants. In contrast to N_2O causing the reduction of stratospheric ozone, NO_x emissions result in the production of ozone in the troposphere (Davidson et al., 2000). Tropospheric ozone is a GHG, an air pollutant effecting human health (e.g. respiratory diseases), and it can damage vegetation (Aneja et al., 2001).

Nitrate leaching is the 'vertical' transport of NO_3^- through soil by water, which can often reach surface and ground water. Increasing NO_3^- levels in drinking water can result in methaemoglobinemia (Di and Cameron, 2002) or 'blue-baby syndrome', although the documented cases of this are extremely rare. This high ingestion of NO_3^- impairs the ability of blood to transport oxygen around the body, where infants are particularly susceptible. It should also be noted, however, that NO_3^- at lower concentrations can also have a beneficial impact on human health (Lundberg et al., 2004). In addition to its human impact, NO_3^- leaching into aquatic environments can lead to eutrophication, algal blooms and death of fish (Di and Cameron, 2002). In a review of 22 studies, average losses of NO_3^- from urine patches amounted to 20% of the applied urine N, although this varies with N loading rate and season of application (Selbie et al., 2015).

The volatilisation of NH_3 can result in aerosol formation, acid deposition and soil acidification (ApSimon et al., 1987; DEFRA, 2002). Deposition of NH_4^+-N can also enrich N-poor habitats, resulting in eutrophication (DEFRA, 2002). In a recent review by Selbie et al. (2015), a mean and range of 12.9 (1-38)% of applied urine-N was volatilised as NH_3 from

ruminant urine patches (n = 59). Sherlock and Goh (1984) measured an NH_3 volatilisation loss of 12-38% of sheep urine N, when applied at a rate of 500 kg N ha^{-1}.

As can be seen from this section, reducing the losses of N from livestock production could yield multiple environmental benefits, the protection of ecosystem services (e.g. clean air and drinking water), greenhouse gas emission reductions and ozone layer protection, as well as conserving N that could fertilise pasture. Due to the cost of applying fertilizers or purchasing animal feed, improvements in N use efficiency can additionally provide an economic benefit for farmers, providing any improvement measures are cost-effective.

2.3 Sheep urine patch characteristics

The simulation of urine events are often required when studying N losses from grazed pastures. This section aims to provide an overview of characteristics relating to sheep urine patches ranging from urine chemical composition and storage of collected urine, urine patch area, urine volume and frequency, and urine-to-soil application methods, in order to understand how best to re-create urine patches for experimental monitoring.

2.3.1 Urine chemical composition

Natural ruminant urine composition can be highly variable due to individual animal's physiology, diet and water intake (Shand et al., 2002; Dijkstra et al., 2013). Sheep urine generally consists of 5-16.9 g N l^{-1} (Sherlock and Goh, 1984; Bristow et al., 1992), and in a recent meta-analysis conducted by Selbie et al. (2015), the average N concentration of sheep urine, from sheep fed a grass-based diet, was reported to be 8.7 g N l^{-1}. The predominant nitrogenous constituent of sheep urine is urea ($CO(NH_2)_2$), which is formed in the liver as a means of detoxifying ammonia in systemic circulation (Spek et al., 2013). Urea comprises the largest fraction of urinary N at 80-90% of the total urinary N excreted (Sherlock and Goh,

1984; Dijkstra et al., 2013). Other nitrogenous constituents at smaller quantities in sheep urine include purine derivatives (allantoin, hippuric acid, uric acid, xanthine and hypoxanthine) creatine, creatinine, amino acids, and NH_3 (Verbic et al., 1990; Bristow et al., 1992).

Purine derivatives arise from the breakdown product of purines (Verbic et al., 1990), as ruminant feed is generally low in purine content, the majority of purine derivatives in ruminant urine originates from rumen microorganisms (Chen and Gomes, 1992). The excretion of purine derivatives has been used as an index of microbial protein supply in ruminants (Chen et al., 1995). The purine bases adenine and guanine, are deaminated to xanthine and hypoxanthine, and mostly converted to uric acid, due to the activity of xanthine oxidase present in the intestine and liver (Tas and Susanbeth, 2007; Dijkstra et al., 2013). Uric acid is subsequently oxidized to allantoin in the liver, and excreted at ca. 4% of urinary-N. Hippuric acid is a conjugate molecule of benzoic acid and glycine, formed for the removal of benzoic acid consumed via aromatic and phenolic constituents within fodder (van Groenigen et al., 2005). In the sheep urine studied by Bristow et al. (1992), ca. 4% of urine N was comprised of hippuric acid. Xanthine, hypoxanthine and uric acid are generally excreted in urine in smaller quantities, where all compounds were present at < 1% of the total excreted N in Bristow et al. (1992). The metabolism of glycine, methionine and arginine produces creatine (ca. 4% of sheep urine N), which can be converted to the cyclic anhydride creatinine (< 1% of sheep urine N) for excretion (Bristow et al., 1992).

Bathurst (1952) investigated the amino acid distribution within the urine of cattle and sheep, where glycine was found to be the major amino acid present. In sheep and cattle urine, total glycine (including bound glycine within hippuric acid and peptides) represented 94% and 98% of the total amino-N respectively. The much lower concentration of other amino acids within the urine suggested that if the bound glycine was in peptide form, it would probably be composed of glycine residues. In contrast, Bristow et al. (1992) found glycine to be prevalent

in sheep urine, however, other amino acids were also found in similar quantities e.g. β-alanine and taurine. In cattle and goat urine, also investigated by Bristow et al. (1992), a dominance of glycine and taurine was observed. Expressed as a percentage of total N, the amino-N fraction has been found to comprise 9.3% (Bathurst, 1952) and 10.5-15.9% (Doak, 1952), however, Bristow et al. (1992) found a much lower value of 0.08-3.67%. As with the other urinary N constituents, the amino fraction also seems highly variable, which is likely to be due to differences in animal diet and physiological status.

The quantity of NH_4^+ in sheep urine, expressed as a percentage of the total urine N, is generally less than 0.5% (Bristow et al., 1992). Nevertheless, urea can be rapidly hydrolysed via the enzyme urease, to yield NH_3 and carbamate as follows (Mobley and Hausinger, 1989):

$$NH_2(CO)NH_2 + H_2O \rightarrow NH_3 + NH_2COOH \qquad \text{(Eqn. 1.1)}$$

The carbamate then spontaneously hydrolyses forming carbonic acid and another NH_3 molecule:

$$NH_2COOH + H_2O \rightarrow NH_3 + H_2CO_3 \qquad \text{(Eqn. 1.2)}$$

The carbonic acid then dissociates forming H^+ and HCO_3^-, and the resulting NH_3 equilibrates with water forming NH_4^+ as displayed by the following equations (Mobley and Hausinger, 1989):

$$H_2CO_3 \rightarrow H^+ + HCO_3^- \qquad \text{(Eqn. 1.3)}$$

$$2NH_3 + 2H_2O \rightarrow 2NH_4^+ + 2OH^- \qquad \text{(Eqn. 1.4)}$$

Initially, urine does not contain urease (Whitehead and Raistrick, 1993) but it can soon become present within urine samples, which can result in decreasing urea contents and increasing NH_4^+ contents if left unfrozen or unacidified. This is because urease is ubiquitous in terrestrial and aquatic environments (Mobley and Hausinger, 1989) and the rate of hydrolysis of urea is dependent on factors which influence the urease-producing bacteria and the activity of the enzyme (Whitehead and Raistrick, 1993). Notably, temperature is an important factor, where higher temperatures would speed up urea hydrolysis due to more favourable conditions for bacterial multiplication and more optimal conditions for enzymatic activity. For these reasons, many authors describe rapidly returning to laboratories with urine samples and advocate freezing samples as soon as possible (Bristow et al., 1992; Whitehead and Raistrick, 1993), or analysing immediately (Shand et al., 2002). Freezing should halt population increase and/or activity of the urease producing microbes (Whitehead and Raistrick, 1993) and analysing immediately would restrict the time taken for microbes to proliferate.

Whitehead and Raistrick (1993) incubated urine from cows at 5, 10, 20 and 35 °C over a period of 21 days to gain information on the factors influencing urea hydrolysis in stored urine. With incubation temperatures over 5 °C, the sum of urea-N and ammoniacal-N were greater than the initial urea-N concentration, indicating that other N containing components were also converted to ammonium (Whitehead and Raistrick, 1993). Knowlton et al. (2010) investigated three collection methods for Holstein heifer urine (chilled, acidified or acidified 6 h after collection) fed either a high or low protein diet. It was found that all three methods performed similarly with regards to N retention, regardless of the protein content of diet (and therefore higher concentrations of urinary N). In order to study the effects of urine deposition on soil, urine should be collected and frozen immediately. Acidifying the urine would alter the pH and would, therefore, not be suitable for investigating transformations of urine compounds within the soil.

In addition to the major nitrogenous constituents of sheep urine, it is also comprised of aqueous salts. Haynes and Williams (1992) analysed the cations and anions within the urine of sheep which were fed either grass/clover pasture or supplements. In both diets, K^+ and Na^+ were the most prevalent cations in the urine, however, sheep on supplements had higher Na^+ than K^+ content and the sheep fed grass had higher K^+ than Na^+ content. This is likely to be due to the initial concentrations of these elements in the feed. Cations present at a lesser extent include Mg^{2+}, Ca^{2+} and NH_4^+. The major anions found within the urine were HCO_3^- and Cl^-, with SO_4^{2-} and NO_3^- in smaller quantities (Haynes and Williams, 1992).

Synthetic urine has been used within experiments on several occasions (e.g. Shand et al., 2000; Shand et al., 2002; Kool et al., 2006; Lucas and Jones, 2006; Hoogendoorn et al., 2008), and is comprised of various quantities of the compounds present in natural urine. Advantages of using synthetic over natural urine include the fact that urine can be difficult to collect (especially if large volumes are required), while synthetic urine provides a convenient medium for experimental use (Shand et al., 2002). Natural urine can be highly variable in terms of N content and caution should be taken when interpreting results from experiments using synthetic urine and microcosm studies, especially if scaling up to pasture scale. Shand et al. (2002) noticed using synthetic sheep urine resulted in an overestimation of results in comparison to natural sheep urine, for example soil pH, dissolved organic C, total dissolved phosphate, dissolved organic N and NH_4^+ and NO_3^- concentrations were all overestimated in this study. Similarly, Kool et al. (2006) compared urine from cattle with three synthetic versions (urea + glycine, urea + hippuric acid and artificial urine with the same nitrogenous composition as the real cow urine) in a soil incubation study. None of the artificial urine studies adequately represented the real urine across all measured variables (N_2O flux, pH, soil respiration and mineral N dynamics) and the use of real urine was advocated wherever possible,

although some artificial urine treatments had similar cumulative N_2O emissions to real urine in the study by Bell et al. (2015).

Sheep urine composition can vary widely as a function of diet, water and physiological status of the animal. Therefore, wherever possible, real sheep urine should be collected for experimental purposes. Ideally the urine collected should reflect the grazing systems and management practices relevant to the area of study e.g. relevant sheep breeds, diets and applying at representative times of the year (to match diet). When collecting urine for field application, samples should be frozen as soon as possible to avoid losses of N. In the case of urine application to soil, urine should not be acidified, as this would change the pH of the urine, and may alter downstream N processing. Artificial urine can provide a useful medium where alterations in N composition are to be examined, but should be made as close in composition to real urine as possible. Further work is required to understand how urine composition influences downstream N_2O producing processes.

2.3.2 Sheep urine patch area

The urine patch is the area where certain nutrients are returned and recycled, or lost, from pastures and often show up in pastures as areas of grass which are greener than others. The area and volume of soil influenced by a urine patch are important parameters to consider when recreating urine patches in the field. The urine patch area has been considered in terms of both the wetted area and the effective area, and when used to model N leaching losses, the volume of soil influenced by urine has been considered as an inverted cone (Fig. 2.2; Li et al., 2012). The wetted area has been defined as the area of soil where urine has been directly voided, and the effective area has been defined as the area where plants can access the nutrient pool within the patch (Lantinga et al., 1987; Selbie et al., 2015). Solutes within the urine patch could move via mass flow (the movement of water and solutes together due to a pressure

gradient) or via diffusion (the movement of solutes due to a concentration gradient), where solute movement via diffusion can occur independently of water (Oyewole et al., 2013).

The effective area is generally larger than the wetted area due to horizontal and vertical diffusion of solutes, and extension of roots into the nutrient rich patch. If a soil has a large infiltration capacity then the wetted area would be expected to decrease, as the urine would tend to travel down the soil profile with minimal lateral spread (Li et al., 2012). What is less clear about this definition of the urine patch area, is the extent of horizontal diffusion of solutes in the urine patch over time and what impact this has on soil processes, N_2O emissions and the measurement of such emissions from urine patches (this is investigated in Chapter 3).

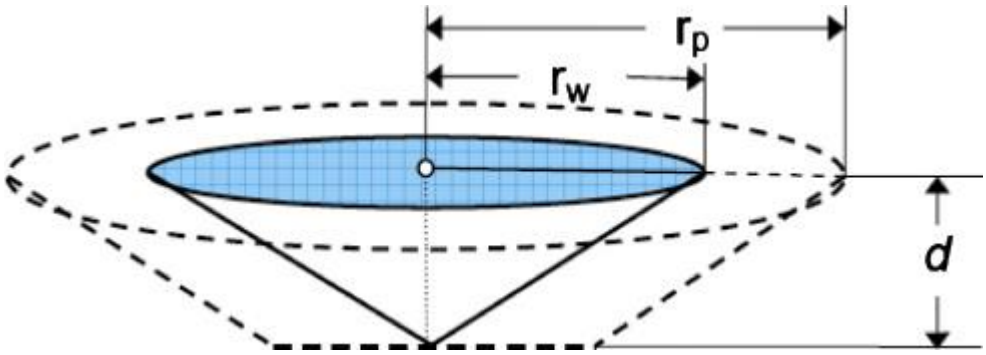


Figure 2.2 Diagrammatic representation of the urine patch area, where r_w represents the wetted area radius, r_p represents the pasture response area radius and d represents the depth of urine infiltration. Source: Li et al. (2012).

Williams and Haynes (1994) used a bromide recovery technique to determine the potential size and distribution of typical sheep (200 ml) and cattle (2000 ml) urination events applied by pouring onto soil, at relevant heights for each animal. As shown in figure 2.3, the depth is shallower and the surface area covered is smaller for the sheep compared to cattle urine, due to the lower volume applied.

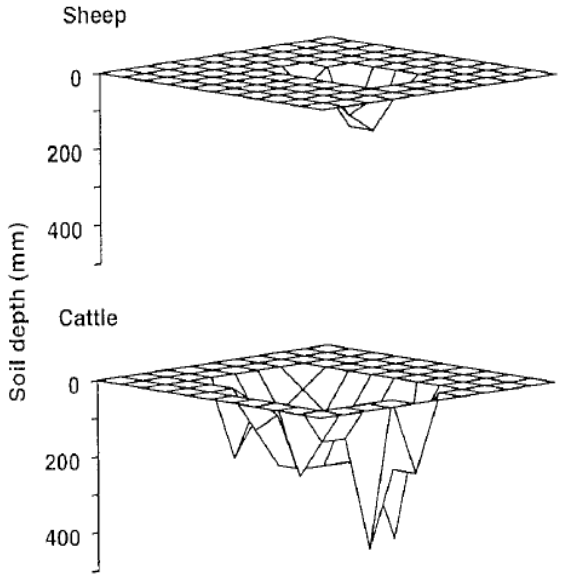


Figure 2.3 Distribution of Br^- from simulated sheep and cattle urine, using potassium bromide solution as a tracer. Source: Williams and Haynes (1994).

In this study, the wetted area of the sheep urine patch applied to a silt loam soil with a *Lolium perenne* L. dominated sward ranged from 0.043-0.055 m^2, and the maximum soil depth reached was 15 cm, with the amount and concentration decreasing with depth. Doak (1952) also provided a value for sheep urine patch area of 300 cm^2 based on a 150 ml urine application to a . The urine patch area is likely to vary due to differences in urinary volume slope, soil type and condition (e.g. compaction) and soil moisture conditions, and differences relating to these factors are relatively understudied (Selbie et al., 2015).

In addition to the urine patch area, the total area of pasture covered by urine events has also been studied. Betteridge et al. (2010b) describe the use of a urine detecting sensor for use on female cattle or sheep, where urine flows over a thermistor, and any urination event is logged as the temperature changes. A GPS unit is also attached to the animal so the location of the urine patch can be recorded. From these data the distribution of urine can be mapped, providing useful information on the spatial distribution of urine patches at pasture scale. Betteridge et al.

(2010b) determined that cattle and sheep deposited ca. 41% and 30% of urine over 5% and 7.5% of the pasture area, respectively. Areas of more intense urine deposition were found in livestock 'camping areas'. This supports the idea of targeting e.g. NO_3^- leaching or N_2O mitigation strategies to certain areas within the field, where urine frequency is highest and soil conditions are more conducive to N_2O production.

Betteridge et al. (2013) describe advances in such sensors, where urine volume, frequency and N concentration can all be measured. These are important parameters to consider as the concentration of N and the volume of each urine event causes urine patches of differing N loading rates. Consideration of the mean urine patch volume and frequency causes differences in modelled NO_3^- leaching losses of 5-10%, when compared to using mean values for urine concentration and volume (Li et al., 2012; Betteridge et al., 2013). A similar phenomenon may also occur when using average urine volumes and N concentrations for predicting N_2O emissions from grazed grasslands.

2.3.3 Sheep urine volume and frequency

Urine volume is mainly influenced by the mineral load and water ingested by the animal (Selbie et al., 2015). Urine volume has been reported to be higher when herbage leaves are wet with rainwater or dew (Doak, 1952). Urine volume can also vary as a response to coping with changes in ambient temperatures (Betteridge et al., 2010a). Typically, individual sheep urine events range between 0.1-0.2 l (Doak, 1952; Haynes and Williams, 1993). Daily sheep urine volume ranged from 0.5-3.0 l in Ledgard et al. (2008), and sheep urine frequency has been reported to range between 13-20 times per day (Doak, 1952; Betteridge et al. 2010a; Betteridge et al., 2010b). More research is required on typical urine volumes and frequencies of sheep urine events, particularly in relation to different breeds, diets and management strategies. An assessment of the individual urine volumes from Welsh Mountain ewes is made in Chapter 7.

2.3.4 Sheep urine-to-soil application methods

The most common method for re-creating urine patches for experimental use is applying urine on an area basis within replicated plots (e.g. the equivalent of 4 l m^{-2}; Kelliher et al., 2014). Urine has been applied to soil evenly using watering cans, where the height of urine deposition has even been considered in some studies e.g. Williams and Haynes (1994). Representing sheep urine patches by applying urine over a large area e.g. 1 m^2, has advantages in that there is a large area of soil which can be utilised for soil sampling and instalment of static chambers for monitoring gas fluxes. Nevertheless, in reality, the size of sheep urine patches are much smaller (0.03-0.05 m^2) and the size of urine patches has been shown to influence N losses (e.g. greater NO_3^- leaching losses from cattle-sized compared to sheep-sized urine patches (McDowell and Houlbrooke, 2009), and lower N_2O emissions due to a more even spread across the pasture deposited in smaller sheep-sized patches compared to larger cattle-sized patches (IPCC, 2006)). Many studies apply urine evenly within the chamber basal area or in lysimeter studies (Koops et al., 1997), yet this may confound measurements by the restriction of diffusion of solutes into surrounding soil and the prevention of root extension into the patch. Applying urine over a fixed area, however, is a sensible approach, as it allows the N loading rate to be calculated. It is recommended that the size of urine patches used in field studies should reflect, as closely as possible, the size of a representative urine patch. Other factors which may influence urine N processing is matching the temperature of urine application, although this has not been studied to our knowledge. It is suggested, that static chambers used for monitoring greenhouse gas emissions from urine patches should be larger than the size of the urine patch, to prevent chamber walls restricting diffusion of soluble N and C into surrounding soil (this is investigated in Chapter 3).

2.4 Changes in soil-microbe-plant properties following urine deposition

2.4.1 Changes in soil properties

This section describes the changes in soil properties that occur as a direct result of urine deposition. Shand et al. (2002) added synthetic sheep urine to soils under field conditions and in sward boxes. The applied urea was rapidly hydrolysed within the soil, following first order kinetics, with a half-life of 16.1 hours. The hydrolysis of urea within soil is generally a fast process, which can be stimulated by hippuric acid, which is also present in urine (Monaghan and Barraclough, 1992). The alkaline products generated during urea hydrolysis generally increase soil pH by up to 3 units (Carter, 2007; van Groenigen et al. 2005). This 1000-fold increase in OH^- is associated with increased organic matter solubility and desorption of anions held on exchange sites (Shand et al., 2002). Following nitrification or NH_3 volatilisation, the pH decreases again (Clough et al., 2010). In the experiment conducted by Shand et al. (2002) the pH had returned to that of control soils by 56 days, however, the maximum pH occurred 2 days following urine addition in the sward boxes, but 7 days following urine addition to the field. The solubilisation of dissolved organic N (DON) and consequences for leaching of DON is also an understudied area for urine-influenced soils (van Kessel et al., 2008; Selbie et al., 2015)

As urea in urine hydrolyses, a large amount of NH_4^+ is generated, increasing as the process progresses. If nitrification proceeds quickly, a sharp rise in concentrations of NO_3^- within the soil will occur (with the associated decrease in NH_4^+). The rate of nitrification is important in determining the potential for loss via processes such as leaching and denitrification (Monaghan and Barraclough, 1992). This is due to the greater mobility of NO_3^- within soils in comparison to NH_4^+, therefore enhancing the probability of the N being leached. Generally, in grassland soils, nitrification proceeds only slowly relative to the rate of urea hydrolysis (Monaghan and Barraclough, 1992).

In addition to a rise in pH, the electrical conductivity (EC) of soil also increases following urine addition (Orwin et al., 2010). This is due to the addition of urinary salts, which lower the osmotic potential of the soil water. Increased levels of P have been demonstrated in soils following urine application. Generally ruminant urine does not contain high amounts of P, therefore, the increase must be soil based (Bertram et al., 2012). Bertram et al. (2012) displayed a decrease in total phospholipid-derived fatty acids (PLFA) count (a structural component of cellular membranes), combined with an increase in soluble P in soils treated with urine. The PLFA count is representative of the living microbial biomass, as upon cell death the PLFAs will rapidly degrade. The release of P-containing cell constituents following cell lysis was posed as a possible explanation for the observed soluble P increase and PLFA decrease. Another explanation for higher concentrations of available P is the increased organic matter solubility (i.e. DOP), following the associated pH increase after urine addition to soil.

Soil dissolved organic C (DOC) increases following urine addition, and subsequently declines back to original values over time (Orwin et al., 2010). Within the urine patch, possible sources of C include the C within the urine itself, increased solubilisation of the soil C due to urine addition, lysis of soil microbial cells due to stress induced changes following urine addition or from the leakage of C from scorched plant roots (Ambus et al., 2007). Carter et al. (2006) and Ambus et al. (2007) tested the hypothesis that urine deposition causes an increase in the release of root-derived C compounds, resulting in more denitrification and N_2O emissions. Both studies concluded that urine did not cause a rapid increase in C release from roots, possibly due to urine-induced root death reducing respiration. Lambie et al. (2012) found that C solubilisation from soil cores amended with cow urine was ten times that solubilised with water alone. The evolution of CO_2 following urine addition to soils exceeded the amount initially present in the urine in studies by Clough et al. (2003) and Kool et al. (2006). This

indicated that priming of soil C release had occurred due to increased soil microbial activity (Kool et al., 2006).

2.4.2 Urine patch and microorganisms

Microorganisms control many of the processes involved with C and N cycling in soils, therefore the effect urine has on the microbial community is an important area of study. The microbial community are responsible for N_2O producing and reducing pathways, and understanding how the individual processes relate to changes in environmental conditions and agricultural management practices is key for the development of mitigation strategies (Smith, 2010). Orwin et al. (2010) highlight the fact that our understanding of the biotic responses to urine addition is still limited. Features of the urine patch can be both stimulatory and inhibitory for microbial activity. Large inputs of N stimulate nitrifier and denitrifier activity (Orwin et al., 2010), and further factors such as the addition of water, labile C and other nutrients could have a stimulatory effect. Hostile conditions of the urine patch include a high pH, high osmotic stress and a build-up of toxic substances / metabolites (e.g. NO_2^- and NH_3; Petersen et al., 2004).

Orwin et al. (2010) conducted an experiment adding bovine urine to soil cores at two differing moisture treatments (35% and 75% water-filled pore space; WFPS). At 75% WFPS, the microbial biomass was often significantly lower than the dry treatment; however, rates of nitrification and denitrification were higher in the wet treatment. Nitrous oxide fluxes were also significantly higher in the 75 % WFPS treatment in comparison to 35 %. It was concluded that moisture content and aeration status had a strong effect on the way soil microbes processed the urine-N (Orwin et al., 2010). Lovell and Jarvis (1996) found the microbial biomass increased in size following urine application; however, this was not significantly different to the water control. Although microbial biomass size was similar, there was an increase in

activity (CO_2 production) and functioning of the soil microbial biomass after urine treatment (Lovell and Jarvis, 1996).

An increase in PLFA yields were observed in an experiment conducted by Petersen et al. (2004), where cattle urine was applied to confined field plots. This increase in the size of the microbial pool occurred over four days, with a shift in community composition. More anteiso fatty acids were produced, which belong to the family of branched-chain fatty acids (Kaneda, 1991). It was suggested that the presence of these fatty acids resulted in an increase in membrane permeability, which may be a stress response to hyperosmotic conditions. Bertram et al. (2012) found little evidence for a direct effect of increasing urinary salt concentrations on the stress of the soil microbial community, however, microbial stress was induced by the addition of the urine in association with a high moisture content. A decrease in PLFAs were observed between days 3 and 8 following bovine urine addition to soil cores in the study conducted by Bertram et al. (2012). This decline occurred at the same time as an increase in available P and was attributed to stress induced lysis of microbial cells, releasing P-containing constituents.

Di et al. (2010) investigated nitrification rates and growth of ammonia-oxidizing archaea and ammonia-oxidizing bacteria in differing soil depths, following urine amendment. The ammonia-oxidizing bacteria were more abundant and had a higher activity in comparison to the ammonia-oxidizing archaea, especially in the surface horizon. The rate of nitrification was attributed to the ammonia-oxidizing bacteria in these New Zealand pasture soils. This has consequences for the design of nitrification inhibition strategies, where this study supports targeted surface application. In this study, additions of urine also stimulated growth of ammonia oxidizing bacteria but not archaea.

The effect of urine deposition on fungal communities has been less well examined than other soil microorganisms. A shift in fungal community structure was observed by Rooney and

Clipson (2009) following the addition of synthetic sheep urine to soil microcosms. Shifts in fungal populations may be observed due to a variety of factors including changes in pH, nutrient availability, and changes in rhizodeposition (Rooney and Clipson, 2009). Jirout et al. (2013) isolated 36 fungal species from cattle overwintering areas, under laboratory conditions 64% of these species were able to produce N_2O. The contribution of N_2O via fungal species under field conditions is not well understood and requires further research.

2.4.3 Urine and herbage

A reduction in root biomass has been reported following urine applications, for example the root biomass of *Agrostis capillaris* and *Lolium perenne* significantly decreased in a pot experiment examining the addition of synthetic sheep urine over time (Rooney and Clipson, 2009). Vegetation may be scorched (necrosis of tissues) following urine additions to soil, and botanical composition may be altered (Shand et al., 2002). Rooney and Clipson (2009) reported scorching becoming apparent 8-10 days following synthetic urine addition to soil microcosms. Ledgard et al. (1982) reported a decrease in clover growth and N fixation due to cow urine addition to pasture.

Herbage biomass production is generally found to increase following urine deposition e.g. Williams and Haynes (1994) reported an increase in herbage dry matter of 63-75% in urine treated plots compared to non-urine treated plots. The N and K content of herbage was found to increase following sheep urine application, due to the high concentrations of these elements in the initial urine samples (Haynes and Williams, 1992). The increased N supply within pastures can result in dark green coloured vegetation above urine patches. The response of vegetation will be larger than the initial wetted area following deposition, due to the roots and stolons of pasture plants which are able to access this pool of nutrients (Li et al., 2012), and due to some diffusion of NO_3^-.

2.5 Terrestrial transformations of N leading to N_2O formation

As the measurement and mitigation of N_2O emissions from sheep urine patches is the major topic of this thesis, this section will discuss the terrestrial N transformations that may occur in urine-influenced soils. Current understanding of the microbial pathways leading to N_2O production have been described in detail in Wrage et al. (2001), Baggs (2008) and Baggs and Philippot (2010). A schematic of the microbial processes leading to N_2O can be seen in Figure 2.4, where ammonia oxidation, denitrification and nitrate ammonification are the microbial N_2O-genic processes in soils. The majority of urine N enters the soil as urea and other small organic N molecules, which mineralize to NH_4^+ in the soil. Mineralisation is the process where soil organic N is converted to NH_4^+/NH_3 and immobilisation occurs when NH_4^+/NH_3 is converted back to organic N. These processes are likely to regulate the supply/removal of reactants for N_2O-producing pathways, however, this section will focus on N transformations which directly lead to N_2O production, therefore mineralisation and immobilisation will not be considered in detail here.

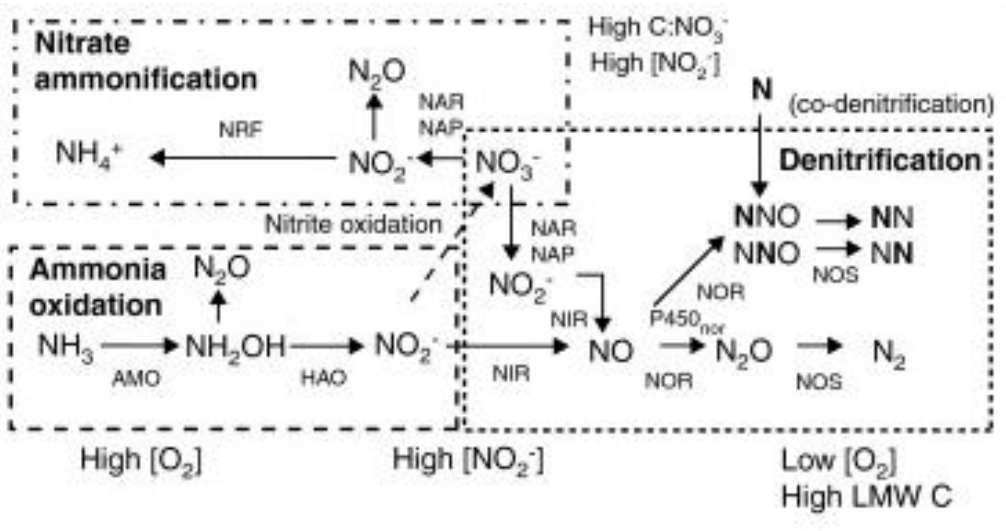


Figure 2.4 The microbial pathways leading to N_2O, with environmental conditions favourable for each process and enzymes regulating each pathway. Source: Baggs (2011).

2.5.1 Nitrification

Biological nitrification is the oxidation of NH_4^+ or NH_3 to NO_3^-, involving two intermediates, hydroxylamine (NH_2OH) and nitrite (NO_2^-). In Fig. 2.4, nitrification is represented by the combination of ammonia oxidation and nitrite oxidation. The first step in nitrification involves oxidation of NH_3 to NH_2OH, which is carried out by primary nitrifiers and catalysed by the enzyme ammonia monooxygenase (AMO; Fig. 2.4; Wrage et al., 2001). Subsequently, the NH_2OH is oxidised to NO_2^-, which is catalysed by the enzyme hydroxylamine oxidoreductase (HAO; Fig. 2.4). Oxidation of NO_2^- to NO_3^- is carried out by the genera *Nitrobacter* (Haynes and Williams, 1993) and catalysed by nitrite oxidoreductase (Wrage et al., 2001). Autotrophic nitrifiers i.e. the process of ammonia oxidation, can be a significant source of NO and N_2O (Lipschultz et al., 1981) e.g. under anaerobic conditions NO_2^-, instead of oxygen, can be used as a terminal electron acceptor where N_2O and NO are produced (FAO, 2001). N_2O can also be formed via the chemical decomposition of the nitrification pathway intermediates (NH_2OH or NO_2^-; Wrage et al., 2001).

Urine-N would undergo nitrification following urea hydrolysis, or the breakdown of other urinary N constituents into NH_3 (e.g. amino acids, purine derivatives). Ammonia oxidation is carried out by Nitrobacteriaceae under aerobic conditions, where maximum activity is observed at 30-60% WFPS (FAO, 2001; Wrage et al., 2001). The known bacterial autotrophs that oxidise NH_3 belong to the genera *Nitrosomonas* and *Nitrosospira* (Smith, 2010). As inputs of N via urine are generally in forms other than NO_3^-, nitrification is an important step to provide the substrate for other microbial processes which utilise NO_3^- (Fig. 2.4; Yamulki et al., 1998). Nitrification was generally thought to be carried out by autotrophic bacteria (Pedersen et al., 1999), yet a range of heterotrophic bacterial and fungal nitrifiers are also known (Wrage et al., 2001; Smith et al., 2010). Heterotrophic nitrifiers can use inorganic

or organic substrates for nitrification (Barraclough and Puri, 1995) and are also able to denitrify under aerobic conditions (Robertson et al., 1989; Wrage et al., 2001).

The process of nitrifier denitrification is described as the reduction of NO_2^- to N_2O by ammonia oxidizers (Kool et al., 2010). Therefore, N_2O arising from ammonia oxidizers can occur during ammonia oxidation or denitrification by the ammonia oxidizers themselves. Baggs (2011) questions the inclusion of this process as a separate N_2O producing process, as it essentially involves the same enzymes used in denitrification. The ability of ammonia-oxidizing bacteria to denitrify appears widespread, therefore, a redefinition of the functioning of this microbial group may be required (Shaw et al., 2006; Baggs, 2011). Wrage et al. (2004) assessed the contribution of nitrifier denitrification in incubation studies under varying grassland conditions, including artificial urine patches. Nitrifier denitrification was found to be a potentially important pathway for N_2O production, as the greater the concentration of artificial urine-N applied the greater the concentration of NO_2^- in the soil. The increased NO_2^- in the soil would increase the substrate available for nitrifier denitrification. The environmental factors which influence this pathway are poorly understood (Smith, 2010), yet increased NO_2^- concentrations and mildly anaerobic conditions have been identified as potential rate regulating processes (Monaghan and Barraclough, 1993). The high pH and NH_3 concentrations within urine patches slow down the oxidation of NO_2^- to NO_3^-, allowing accumulation of NO_2^- (Monaghan and Barraclough, 1993). It is therefore concluded that nitrifier denitrification is a potentially important source of N_2O in urine patches, but further work utilising natural urine and field conditions will be required to determine its importance under a variety of ecological conditions.

2.5.3 Biological denitrification

Denitrification involves the reduction of NO_3^-, to NO_2^-, NO, N_2O and N_2 (Fig. 2.4). The process is mainly carried out by diverse genera of aerobic heterotrophic microorganisms, which are abundant in soils (Lloyd, 1993). The enzymes (and genes coding for these enzymes) which catalyse the denitrification pathway include nitrate reductase (NAR in Fig 2.4; *napA* and *narG* genes), nitrite reductase (NIR in Fig 2.4), nitric oxide reductase (NOR in Fig 2.4; *nirS* and *nirK* genes), and nitrous oxide reductase (NOS in Fig 2.4; *nosZ* gene; Hochstein and Tomlinson, 1988; Smith, 2010). The genes coding for these enzymes have been used in studies to determine the environmental factors which influence denitrifier community composition (Smith, 2010).

The facultative anaerobes involved in denitrification are able to utilise NO_3- as a terminal electron acceptor under low O_2 conditions (Wrage et al., 2001). Provided there is sufficient NO_3^- and readily available C, under low O_2 conditions, N_2O can be released as an intermediate (Wrage et al., 2001). Smith (2010) identifies the main controlling influences on denitrifier activity as availability of C, O_2 status of the soil (influenced by WFPS), availability of N, temperature and pH. The addition of urine to soil influences all of the factors which affect denitrifier activity. Lloyd (1993) highlights the fact that initially, denitrifiers require aerobic conditions for efficient biomass production, however, rapid denitrification occurs under low O_2 conditions. This may be important upon consideration of grazing livestock urination events, which may increase anaerobic conditions in localised areas previously unaffected by low O_2 levels. Denitrification has been found to be highly spatially variable, due to the variable nature of identified controlling factors of denitrification, including anaerobic soil microsites (FAO, 2001). The denitrification process occurs at particularly high rates in soil which has 50-90% WFPS (FAO, 2001).

The process of co-denitrification (Fig. 2.4) involves the utilisation of one molecule of N from a co-substrate (e.g. NH_3, amino acids, aniline, azide) combined with one molecule of

N from NO or N_2O, potentially forming two molecules of N_2O compared to one via the normal denitrification process (Su et al., 2004; Baggs, 2011). The significance of this pathway in terms of N_2O emissions from soil is yet to be established.

2.5.4 Nitrate ammonification

Nitrate ammonification (Fig. 2.4), also termed dissimilatory nitrate reduction to ammonia (DNRA), is the process where NO_3^- is reduced to NO_2^-, followed by NH_4^+ (Fig. 2.4; Smith, 2010). This process is carried out by fermentative bacteria and is less well characterised in soils than other N transformations. The major environmental drivers governing its occurrence in soils include the redox status, C/NO_3^- ratio, pH, bulk density, sand content and NO_2^- concentration (Wan et al., 2009; Rütting et al., 2011). The process is thought to conserve N, as NO_3^- is converted to the less mobile NH_4^+. N_2O can also form during this anaerobic process, which may occur concurrently with denitrification (Stevens et al., 1998). Nitrate ammonification is favoured under high pH levels, which would occur initially under a urine patch scenario. Following urea hydrolysis and nitrification in a urine patch, NO_3^- would not be limiting, therefore, competition for C would determine whether denitrification or nitrate ammonification would occur (Tiedje, 1988).

2.5.5 Chemodenitrification

Chemodenitrification is attributed to any non-biological source of NO, N_2O or N_2 (Smith, 2010), therefore, is not displayed in Fig. 2.4. This process is generally favoured under high NO_2^- concentrations and a pH of < 5.5 (Haynes and Williams, 1993). An alkaline pH is usually present initially within a urine patch, therefore, chemodenitrification reactions are expected to be of minor importance for N_2O emissions under this scenario, unless dealing with very acidic soils.

2.6 Measurement of N_2O emissions from urine patches

The major challenges associated with measuring N_2O includes the low atmospheric concentration of the gas (320 ppb), and the episodic nature of N_2O emissions, with large flux variations observed both spatially and temporally (Rapson and Dacres, 2014). This section explores methodological, analytical and modelling approaches to quantifying N_2O emissions from soils. The static chamber technique, combined with analysis of headspace gas samples via gas chromatography (GC) equipped with an electron capture detector (ECD) is one of the most widely used, versatile and low-cost technologies used for N_2O flux measurements (Hutchinson and Mosier, 1980; de Klein and Harvey, 2012), which has been in use for over 40 years (Hensen et al., 2013), resulting in a wide range of largely comparable data sets. This technique was utilised in the experimental chapters of this thesis, via both manual and automated campaigns.

The optimisation and need for standardisation of the static chamber technique for monitoring N_2O fluxes has been recognised (de Klein and Harvey, 2012), where important considerations when using this technique include chamber design, deployment, sampling frequency and time of day to sample. The calculation of fluxes can either be conducted by assuming linearity in the increase of chamber headspace concentrations, or by using non-linear or best-fit model approaches (Kroon et al., 2008; Pederson et al., 2010; Hensen et al., 2013). Prioritising increased chamber numbers to account for spatial variability in N_2O fluxes, rather than prioritising increased number of samples per chamber closure period has been suggested by Chadwick et al. (2014), due to the far greater uncertainty introduced form spatial variability in fluxes, compared with either reducing the number of headspace samples or the assumption of linearity of headspace concentrations. The final strategy used, however, is likely to be a compromise between the necessary requirements of the experiment, and time and resources available for specific projects. Automated chamber systems reduce manual labour and provide

enhanced temporal resolution, although chamber numbers and spatial area coverage are generally limited.

In order to improve and develop current or new mitigation strategies for N_2O emissions from soils, quantifying the microbial sources of N_2O production will be necessary (Köster et al., 2013). Previous techniques which have been utilised to apportion N_2O production to different sources (e.g. nitrification or denitrification) include the use of process-specific inhibitors such as C_2H_2, or isotopic labelling techniques and natural abundance techniques ($\delta^{15}N$ and $\delta^{18}O$; Baggs et al., 2008; Decock and Six, 2013; Wolf et al., 2015). Novel approaches to source partition N_2O emissions from soils include the measurement of N_2O isotopomers i.e. the distribution of ^{15}N in N_2O as either $^{14}N^{15}N^{16}O$ ($^{15}N_\alpha$) or $^{15}N^{14}N^{16}O$ ($^{15}N_\beta$; Toyoda and Yoshida, 1999; Decock and Six, 2013). The site preference ($SP = \delta^{15}N_\alpha - \delta^{15}N_\beta$) of N_2O has been shown to differ during differing N_2O production pathways, therefore, this technique is gaining interest as a tool for identifying the source of N_2O from soils. Iotopomers of N_2O have been analysed using Fourier transform infrared mass spectroscopy (FTIR-MS) and, more recently, quantum cascade laser based absorption spectroscopy (QCLAS; Waechter et al., 2008; Wolf et al., 2015). The latter is more applicable for continuous measurements under field conditions and has been found to have a higher precision for SP values in comparison to FTIR-MS (Köster et al., 2013).

Tower-based micrometeorological techniques for measuring N_2O emissions are conducted over larger areas (>10 m^2 to field and regional scale) and, therefore, avoid some of the issues related to spatial heterogeneity when utilising the chamber technique (Pattey et al., 2007; Phillips et al., 2007; Hensen et al., 2013). The range of micrometeorological techniques is beyond the scope of this review, however, it is extensively reviewed in Skinner and Wagner-Riddle (2012). Advances in optical techniques for measuring N_2O (e.g. QCLAS) are providing excellent performance, sensitivity and reliability for tower-based N_2O flux measurements,

however, such instrumentation is much more costly than a GC-based system (Hensen et al., 2013). Modelling techniques have also been used to estimate N_2O emissions and NO_3^- leaching from grazed pastures (Saggar et al., 2007; Giltrap et al., 2010; Cichota et al., 2013). Process based models are important for estimating N_2O emissions from soils under differing land use types and future climate change scenarios, where micrometeorological techniques are useful for validating process based models (Eugster and Zeemun, 2006; Pattey et al., 2007).

The Intergovernmental Panel on Climate Change (IPCC) provide a three tier methodological approach to estimating N_2O emissions, for national inventory purposes. The Tier 1 approach uses a default emission factor (1% of applied N for sheep and 2% for cattle) in combination with activity data (e.g. number of grazing animals; IPCC, 2006). This is a simplistic approach, which does not consider confounding factors such as climatic conditions, soil type or differences in management (IPCC, 2006). The Tier 2 approach uses country specific data and the Tier 3 approach is based on modelling. Estimates of N_2O emissions from varying sectors are important in determining where mitigation options should be focused, however, individual source estimates of N_2O emissions are still considered to have a high uncertainty (Ambus et al., 2007; IPCC, 2007). The accuracy of such estimates may decrease if C and N interactions are not adequately represented under changing climatic conditions (Gärdenäs et al. 2011). Increasing regional field-scale measurements of direct and indirect N_2O emissions will improve the accuracy of country specific emission estimates. In order to provide more accurate emission data, a move to the Tier 2 and Tier 3 approach is warranted.

2.7 Nitrification inhibitors as tools to mitigate sheep urine patch N_2O emissions

Several differing mitigation strategies have been proposed for reducing N losses from grazed pastures, which have been reviewed extensively in Eckard et al. (2010) and Luo et al. (2010). In this Thesis, the use of nitrification inhibitors were selected for study, as in principal,

they appear to be an attractive strategy to reduce both NO_3^- leaching and N_2O emissions from urine patches or fertilizer applications, whilst also potentially providing biomass yield increases (Di and Cameron, 2007; Abalos et al., 2014). Increasing the residence time of NH_4^+ increases opportunity for plant acquisition, immobilization and adsorption (Di and Cameron 2007), thus reducing the NO_3^- supply available for leaching and denitrification processes. If more of the added N remains as NH_4^+ plant use efficiency can increase and losses via leaching and denitrification may be reduced (Cookson and Cornforth, 2002). Such additional benefits may become important in terms of reducing the amount of N lost as N_2O from agricultural soils (e.g. 1% of that applied in sheep excreta), as this does not represent a significant economic loss to farmers. However, combined with the reduction of other N losses (e.g. NO_3^- leaching), nitrification inhibitors become a more attractive strategy for improving agricultural N use efficiency. In addition, nitrification inhibitors may reduce N_2O emissions from two biological N_2O production pathways, from the process of nitrification itself, and by reducing the amount of substrate available for denitrification.

The first and rate-limiting step of nitrification, the oxidation of NH_4^+ to NO_2^- (Zerulla et al., 2001; Moir et al., 2007), is inhibited by a wide variety of compounds of biological (e.g. root exudates; Subbarao et al., 2007; Zakir et al., 2008; Subbarao et al., 2009) and synthetic origin (e.g. dicyandiamide (DCD), 3,-4-dimethylpyrazole-phosphate (DMPP), several heterocyclic N compounds; McCarty and Bremner, 1989; Zerulla et al., 2001). Ammonia monooxygenase has a broad range of substrates which it can catalytically oxidize, therefore, many nitrification inhibitors work on the basis of competing for the active site of this enzyme (McCarty, 1999). Although several compounds which inhibit nitrification have been identified, this thesis focuses on two of the most widely used nitrification inhibitors in agriculture, DCD and DMPP (Liu et al. 2013). DCD blocks the electron transport chain in the cytochrome of ammonia monoxygenase (AMO), whereas DMPP binds indiscriminately to the membrane-

bound AMO (Chaves et al. 2006; Fiencke and Bocke 2006; Benckiser et al. 2013). No significant toxicological effect on soil bacterial communities have been found for DCD applied to soils (O'Callaghan et al., 2010; Wakelin et al., 2013), and any effects have been shown to be minimal and transient (Morales et al., 2015). DMPP has also been the subject of extensive ecotoxicological tests, required for European Law, and is not recognized as a hazardous substance (Zerulla et al., 2001). Studies indicate that both DCD and DMPP effect ammonia-oxidizing bacteria, but have a limited effect of ammonia-oxidizing archaea, suggesting that ammonia oxidation could still take place form this consortia of soil microorganisms, following nitrification inhibitor addition to soils (O'Callaghan et al., 2010; Di et al., 2010; Kleineidam et al., 2011).

The nitrification inhibitor DCD has been researched extensively for use within livestock urine patches, and has even been used in commercial pastures in New Zealand (O'Callaghan et al., 2010; Liu et al., 2013). Different application methods of DCD have been studied for use in urine patches, typically DCD is sprayed directly onto pasture soil (Moir et al., 2007), however, oral administration to cattle (O'Connor et al. 2013; Welten et al. 2013), infusion of DCD into the rumen or abomasum of sheep (Ledgard et al. 2008), incorporation into fertiliser granules and addition of DCD within a biodegradable hydrogel to slow its release in soil (Minet et al. 2013) have also been investigated. The blanket approach to DCD application is generally inefficient, as the majority of N within older urine patches would have already been converted to NO_3^- (Zaman and Nguyen, 2012). Targeting urine patches within space and time e.g. infusing DCD into the gastrointestinal tract of sheep (Ledgard et al., 2008), or applications to livestock camping areas, would allow enhanced efficiency of nitrification inhibitors and would reduce the overall cost in comparison to broadcast applications (Luo et al., 2015).

Typical DCD application rates are 10 kg DCD ha^{-1} (Moir et al., 2007; O'Connor et al., 2012a; O'Connor et al., 2012b), although higher application rates (up to 60 kg DCD ha^{-1}) have

been suggested for increased efficacy (Luo et al., 2015). In intensive sheep winter grazing systems in New Zealand, DCD (10 kg ha^{-1}) was found to reduce N_2O emissions and NO_3^- leaching from late autumn applied urine patches (300 kg N ha^{-1}) by 72% and 70%, respectively (Moir et al., 2010). In hill-grazed pastures, DCD reduced sheep urine patch N_2O emissions by 40-80% (Hoogendoorn et al., 2008). No significant effect of DCD was found for NO_3^- leaching from sheep urine patches applied to a winter forage crop, although the amount leached was generally three times lower than that from cattle urine patches (McDowell and Houlbrooke, 2009). Moir et al. (2007) demonstrated that a fine particle suspension of DCD, applied at a rate of 10 kg ha^{-1}, was effective at reducing NO_3^- levels from cattle urine patches (1000 kg N ha^{-1}) from 40-80 kg N ha^{-1} to 10 kg N ha^{-1}. Forage dry matter production was also found to increase by at least 20% annually in the DCD treatments, over the course of the field trial. The results of O'Connor et al. (2012a; 2012b), however, suggest that applying DCD (10 kg ha^{-1}) does not consistently increase herbage production in Irish grassland systems.

DMPP has been less well studied for use within urine patches, in comparison to DCD. This is likely to be due to the development of this product as a fertilizer coating, marketed as ENTEC (developed by BASF; Pasda et al., 2001; Zerulla et al., 2001). Typically, DMPP has been applied at application rates ten times lower than that of DCD, at 0.5-1 kg DMPP ha^{-1}, which may result in a lower cost of application for farmers. It has also been demonstrated to have a lower phytotoxicity in comparison to DCD (Zerulla et al., 2001). Di and Cameron (2011) found a significant reduction in the ammonium oxidation rate constant in cattle urine-influenced (1000 kg N ha^{-1}) pastures in New Zealand, following a liquid application of DMPP. In a lysimeter study conducted by Di and Cameron (2012), a liquid DMPP application (5 kg DMPP ha^{-1}) was found to be just as effective as DCD (10 kg DCD ha^{-1}), at reducing N_2O emissions (by 62-66%) and NO_3^- leaching from a 1000 kg N ha^{-1} cattle urine application. Misselbrook et al. (2014) and Barneze et al. (2015), however, found that pyrazole derivatives

(similar in structure to DMPP) did not significantly reduce N_2O emissions from cattle urine, applied under UK summer conditions.

The fate of nitrification inhibitors applied to soil is important, as this will regulate the amount of active compound available in the soil, and the length of time they are effective in reducing N losses (O'Connor et al., 2012a). The effect of temperature and moisture on the persistence of both nitrification inhibitors is fairly well described. DCD is most effective when applied at temperatures of 10ºC or less (Kelliher et al., 2008) and a decrease in efficiency of DCD may also be observed under high rainfall, where the compound may be leached below the grassland rooting zone (O'Connor et al., 2012a). DMPP performance has also been found to be best under cold and wet conditions (Menéndez et al., 2012). Differences in efficacy between these two nitrification inhibitors has been attributed to a lower mobility of DMPP in comparison to DCD, due to a greater sorption of DMPP (Wissemeier et al., 2001; Zerulla et al., 2001; Di and Cameron, 2012). The potential for spatial separation of the nitrification inhibitor from NH_4^+ is another suggestion for explaining differences in their efficacy (Ruser and Schulz, 2015). However, few studies directly compare these processes for DCD and DMPP, especially for urine-influenced soil. In Chapter 5, a comparative assessment of the mobility and co-location of DCD and DMPP with urine-derived NH_4^+ is made, in a range of soils of contrasting texture and organic matter. The factors which may influence persistence of the compounds within the soil are also compared, e.g. sorption, microbial uptake and microbial degradation.

Despite the promising reductions in N_2O emissions and NO_3^- leaching observed by application of DCD to urine patches, in 2012, the use of DCD was suspended for sale and use in New Zealand (Ministry for Primary Industries, 2013). This was due to the finding of milk products contaminated with DCD, when nitrification inhibitors were applied to commercial dairy pastures (Kim et al., 2012; Chen et al., 2014). There is limited information, however,

regarding the mechanism of DCD entry into milk products. In Chapter 6, the potential for direct plant acquisition of this nitrification inhibitor is assessed.

Of the limited assessments of DMPP, and similar compounds, applied to ruminant urine patches, its effectiveness appears inconclusive. Further research is required to determine the efficacy of DMPP under differing edaphic and environmental conditions. Whether DMPP is effective at reducing N_2O emissions from sheep urine patches has also not been studied. In Chapter 7, the effectiveness of DMPP applied to sheep urine patches under UK summer conditions is assessed, varying the time-since DMPP application.

2.8 Conclusions

Urine patches in grazed grasslands can be sites of significant N loss (as N_2O, NO_x, NO_3^- and NH_3), resulting in the contribution to detrimental environmental, human health and economic impacts. Reducing agricultural inputs of reactive N may pose a difficult challenge under increasing intensity, yet it could ultimately lead to a wide range of benefits spanning resource efficiency and human and ecological health. Understanding the spatial and temporal dynamics of C and N interactions in the urine patch could feed into modelling systems, enabling accurate quantification of N loss pathways, under a range of environmental parameters. The best mitigation options are likely to take into account the spatial and temporal variability of N_2O emissions from urine patches, therefore, understanding these processes are important for the development of optimal mitigation strategies.

Further work is required to understand how urine patch parameters can influence N_2O production e.g. determining the importance of including urine patch diffusional areas within chambers (addressed in Chapter 3) and determining how urine patch size and N concentration influence N_2O emissions, within typical ranges reported for sheep (addressed in Chapter 4). The efficacy of nitrification inhibitors in reducing N_2O emissions has been found to vary

widely. Further research is required to determine factors influencing nitrification inhibitor removal/degradation processes in contrasting soils (addressed in Chapters 5 and 6) and to determine whether DMPP is effective at reducing N_2O emissions from sheep urine patches under summer conditions in the UK (addressed in Chapter 7).

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Chapter 3

The urine patch diffusional area: an important N_2O source?

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KAM, DLJ and DRC designed and conceived the experiment, KAM conducted the practical work, analysed the data and prepared the manuscript.

Abstract

Urine patches contribute greatly to greenhouse gas emissions within livestock-grazed ecosystems. The effective area of a ruminant urine patch comprises the wetted area, the diffusional area and the pasture response area. This study specifically assesses the importance of considering the diffusional area for monitoring urine patch N_2O emissions. Spatial and temporal changes in N_2O emissions and potential drivers of emissions (soil pH, EC, redox potential, dissolved organic carbon and nitrogen, NO_3^- and NH_4^+) were measured in sheep urine amended Eutric Cambisol mesocosms, maintained at 50% or 70% water-filled pore space (WFPS). At 70% WFPS, over 10 weeks, the emission factor (EF) was greater when considering the wetted area plus a 9 cm diffusional area (EF = 2.75 ± 0.72% of applied N) than when considering the wetted area alone (EF = 1.44 ± 0.30% of applied N); differences were not statistically significant at 50% WFPS. Redox potential, total extractable N and WFPS contributed significantly to the observed variation in daily N_2O fluxes from the urine patch. We conclude that the urine patch diffusional area is an extremely important source of emissions from urine patches. This has implications when measuring EFs, as the lateral diffusion of solutes may be restricted by chamber walls resulting in an underestimate of N_2O emissions, particularly at higher soil moisture contents. Site-specific assessments of the urine patch diffusional area should be made, and accounted for, prior to monitoring emissions and calculating emission factors from urine patches applied within chambers.

Key Words: animal waste; denitrification; DOC; DON; nitrous oxide; grazing returns

3.1 Introduction

Grazing returns of excreta to pasture soils are estimated to account for 40% of the total (direct and indirect) nitrous oxide (N_2O) emissions from animal production systems, globally (Oenema et al., 2005). Additions of labile carbon (C) and nitrogen (N) to soil in the form of urine (van Groenigen et al., 2005) fuel the major microbial N_2O producing processes of nitrification and denitrification, creating "hot spots" and "hot moments" for emissions within pastures (McClain et al., 2003; Groffman et al., 2009). The current default IPCC emission factor used in national inventories for excretal deposition to soils are 1% and 2% of deposited N for sheep and cattle, respectively (IPCC, 2006); yet, this Tier 1 approach lacks accuracy as it fails to account for variation in N_2O emissions due to environmental, edaphic or management related factors (Skiba and Smith, 2000; Skiba et al., 2012; Buckingham et al., 2014).

Variability in N_2O emissions from urine patches can arise due to differences in urine composition, the amount of N excreted and the volume and frequency of urine events (Dijkstra et al., 2013). Additionally, microbial N_2O production and consumption processes depend on several interacting environmental controls (Bouwman et al., 2013) such as N supply, soil temperature, soil moisture, oxidation-reduction potential (ORP), the availability of labile organic compounds, soil type, soil pH and climate (Skiba and Smith, 2000; Butterbach-bahl et al., 2013). The ORP conditions can affect the chemical status of soil nutrients and affect the activity of microorganisms, and this parameter is generally negatively correlated with pH and EC and can be used to indicate the aeration status of soils (Husson, 2013). Urine patches offer potential for emission reductions and improvements of nitrogen use efficiency (NUE) within the agricultural sector, yet a greater understanding of the spatial and temporal variability in N_2O emissions from urine patches (at several scales of magnitude) is required to improve emission estimates and provide information for emission reduction strategies, such as the use of nitrification inhibitors.

The urine patch "wetted area", where urine is directly voided, has been distinguished from the "effective area" which incorporates the diffusive edge of solutes and the plants able to access this pool of nutrients via root extension (Selbie et al., 2015). It is suggested that the effective area actually comprises the "wetted area", the "diffusional area" and the "pasture response area" in order to distinguish between these regions. The pasture response area can extend to twice the initial wetted area (Doak, 1952), however, the diffusive edge of urinary N has been shown not to exceed 20 cm beyond the initial wetted area in three soil types (granitic Brunisol, Neoluvisol and Calcosol; Decau et al., 2003). The diffusional area may vary with urinary volume, solute concentrations, soil texture (relating to tortuosity and cation exchange capacity), soil moisture content, topography and time. The pasture response area is likely to be dependent on the magnitude of the diffusional area, the vegetation type and the corresponding root architecture.

Dennis et al. (2011) maintain that for investigating soil nutrient cycling processes, the wetted area is more important than the pasture response area, however, the diffusive edge of solutes may also be important to consider. An overestimation of NO_3^- leaching losses from urine applied to lysimeters may occur if no room is allowed for the diffusional area (Selbie et al., 2015). Similarly, underestimations of N_2O emissions may occur if the urine patch diffusional area is not considered (e.g. applying urine uniformly to the entire area beneath a static chamber for gas flux measurements), due to chamber walls preventing the lateral movement of solutes into surrounding soil. Koops et al. (1997) have demonstrated that N_2O losses from the diffusive zone of an artificial urine patch, applied to a peat grassland, can reach the same order of magnitude as the area where urine was directly applied.

This experiment was predicated on the need to assess the importance of the urine patch diffusional area for different solutes, N_2O-regulating soil properties (e.g. dissolved organic C and ORP) and the accuracy of N_2O emission measurements. Eutric Cambisol mesocosms,

amended with sheep urine, were established in order to 1) assess the spatial and temporal changes in soil properties in the wetted and diffusional area, 2) identify those soil properties which are key drivers of N_2O emissions under two moisture regimes, and 3) compare the N_2O emission factor from the wetted area with that of the wetted and diffusional areas combined. Two soil moisture regimes (50% and 70% WFPS) were included, as this was considered important with regards to both N_2O production processes, emissions and the spatial distribution of solutes within the urine patch.

3.2 Materials and Methods

3.2.1. Soil sampling and analysis

Independent replicate (n = 4) samples of a Eutric Cambisol (0–10 cm) were collected from a sheep-grazed, fertilised grassland located at Henfaes Agricultural Research Station, Abergwyngregyn, North Wales (53°14′N, 4°01′W). After collection, the soil was sieved through a 10 mm mesh. Soil moisture content was determined by oven drying (105 ºC, 24 h), and organic matter was determined by loss-on-ignition (450 °C, 16 h; Ball, 1964). Soil pH and electrical conductivity (EC) were measured using standard electrodes submerged in 1:2.5 (w/v) soil-to-distilled water suspensions. The oxidation-reduction potential (ORP) was measured directly in the soil using an ELIT 31C ORP combination electrode (EA Instruments Ltd., London, UK) connected to a mV reader.

Total soil C and N were determined on oven-dried, ground soil using a TruSpec® Analyzer (Leco Corp., St. Joseph, MI). Within 24 h of soil collection, 1:5 (w/v) soil-to-0.5 M K_2SO_4 extractions were performed; the total dissolved C and N (mineral and organic) in the resulting extracts were determined with a Multi N/C 2100S Analyzer (AnalytikJena, Jena, Germany). Nitrate (NO_3^-), ammonium (NH_4^+) and phosphate (P) within the 0.5 M K_2SO_4 extracts were measured by the colorimetric methods of Miranda et al. (2001), Mulvaney (1996)

and Murphy and Riley (1962), respectively. The cations (Na, K and Ca) within 1:5 (w/v) soil-to-1 M NH_4Cl soil extracts were measured using a Sherwood Model 410 Flame Photometer (Sherwood Scientific Ltd, Cambridge, UK). A summary of the soil characteristics is provided in Table 3.1.

Table 3.1 Properties of the Eutric Cambisol used to fill soil mesocosms. Values represent means ± SEM (n = 4) and results are reported on a dry weight basis.

Eutric Cambisol properties	
Texture	Sandy Clay Loam
Field wet bulk density (g cm^{-3})	1.57 ± 0.05
Moisture content (%)	21.9 ± 1.00
pH	6.91 ± 0.17
EC (µS cm^{-1})	65.4 ± 5.14
ORP (mV)	368 ± 10.3
Total C (%)	3.29 ± 0.22
Total N (%)	0.26 ± 0.15
C:N ratio	13.0 ± 0.99
Dissolved organic C (mg C kg^{-1})	102 ± 8.66
Total dissolved N (mg N kg^{-1})	13.8 ± 1.89
Extractable NO_3^- (mg N kg^{-1})	2.28 ± 0.32
Extractable NH_4^+ (mg N kg^{-1})	0.41 ± 0.24
Extractable P (mg P kg^{-1})	9.27 ± 0.91
Exchangeable Na (mg kg^{-1})	54.0 ± 5.98
Exchangeable K (mg kg^{-1})	181 ± 21.5
Exchangeable Ca (g kg^{-1})	1.09 ± 0.05

3.2.2. Urine collection and analysis

Welsh Mountain ewes (n =5) were fed a diet of freshly cut grass (*Lolium perenne* L.; 80%) and white clover (*Trifolium repens* L.; 20%). Ewes were housed in individual pens on plastic slatted flooring designed for sheep (Rimco Ltd., Yorkshire, UK), with collection trays located beneath the flooring for urine collection. Urine samples were centrifuged (4000 *g*; 10 min) and immediately frozen (-20°C) until required, to minimize losses of N. The urine

collected from five replicate sheep was bulked, in order to provide sufficient urine of a homogenous composition for experimental use. The total dissolved C, total dissolved N, pH, EC, NO_3^-, NH_4^+, P and cations were determined directly within the urine samples, as described for the soil samples. The urea content of the urine was determined via the method of Orsonneau et al. (1992). A summary of the urine characteristics is provided in Table 3.2.

Table 3.2 Properties of sheep urine, applied to Eutric Cambisol mesocosms. Values represent means ± SEM (n = 3), where replicates are analytical replicates of urine combined from 5 individual sheep.

Urine properties	
pH	9.15 ± 0.01
EC (mS cm^{-1})	14.1 ± 0.20
Dissolved organic C (g C l^{-1})	6.03
Total N (g N l^{-1})	3.86
Urea (g N l^{-1})	2.71 ± 0.61
NH_4^+ (mg N l^{-1})	129 ± 5.30
NO_3^- (mg N l^{-1})	1.08 ± 0.02
P (mg P l^{-1})	11.6 ± 0.34
Na (mg l^{-1})	692 ± 1.59
K (g l^{-1})	4.00 ± 0.05
Ca (mg l^{-1})	48.4 ± 0.54

3.2.3 Soil mesocosm preparation, treatment application and sampling regime

Briefly, 5 kg of fresh soil (n = 4) was weighed into polypropylene trays (internal height: 11 cm, internal length: 35.5 cm, internal width: 26.5 cm; see Appendix 1, Fig. 1), and repacked to a depth of 5 cm, resulting in a fresh bulk density of 1.10 g cm^{-3}. Bare pasture soil mesocosms were used, in order to gain a mechanistic understanding of processes that occur at the soil-urine interface, in the absence of competing factors (e.g. plant removal of nutrients, NO_3^- leaching). The soil mesocosms were wetted with distilled water using a fine mist sprayer to facilitate even

coverage to achieve 50% and 70% water-filled pore space (WFPS), where the initial starting weights were recorded. The mesocosms were pre-incubated in a greenhouse maintained at 20°C for 1 week before application of urine, to ensure any observed effects were not due to soil disturbance (e.g. sieving). Soil mesocosms were maintained under these conditions for the duration of the experiment, and rewetted weekly with distilled water to achieve initial starting weights using a fine mist sprayer. Urine (36 ml) was applied in a strip (24 cm × 3 cm) across the width of the mesocosms, resulting in an equivalent urine-to-soil surface area for an average sheep urine deposition (150 ml over 300 cm^2; Doak, 1952). This urine application resulted in an equivalent total N loading rate of ca. 200 kg N ha^{-1}, where other studies investigating sheep urine patches have used N application rates of ca. 300 kg N ha^{-1} (Haynes and Williams, 1993; Moir et al., 2003). Soil was sampled at increasing distances away from the centre of the urine patch, along a horizontal diffusional gradient. Briefly, 0-3 cm represents the centre of the urine patch, with further sampling conducted at 3-6, 6-9, 9-12, 15-18 and 27-30 cm away from the direct area of urine application, hereafter referred to as zone A, B, C, D, E and F, respectively (see Fig. 3.1). The final sampling distance (27–30 cm; Zone F) was considered to be the control, as we hypothesised that this zone would receive no effect from the urine application. Samples were taken from three parallel mesocosms for each replicate (n = 4), to provide enough soil sampling points for the duration of the experiment (10 weeks). Sampling was conducted three times a week for the first two weeks and once a week thereafter, until the end of the experiment.

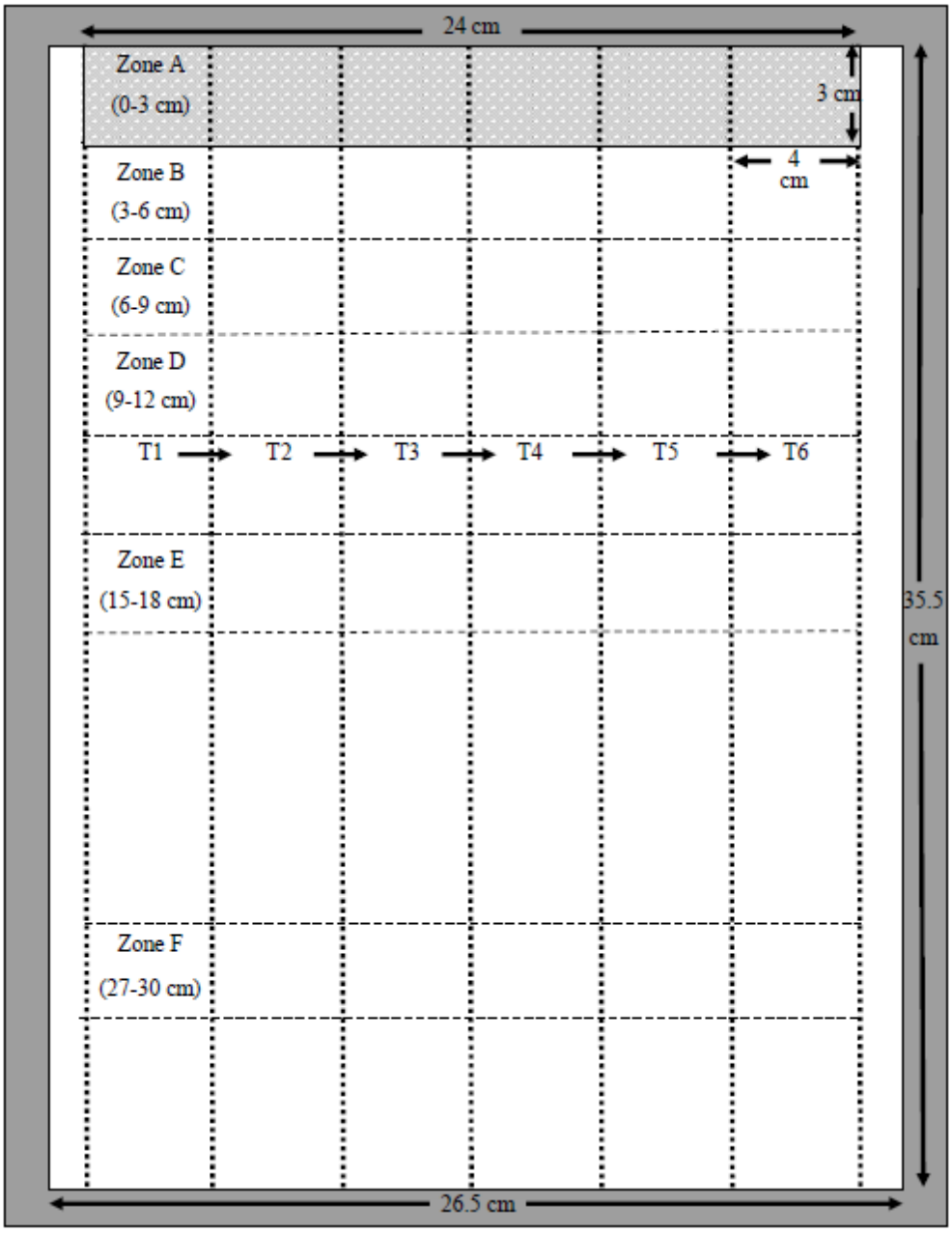

Figure 3.1 Aerial schematic view of the Eutric Cambisol mesocosms, repacked to a depth of 5 cm. The shaded region represents the area of direct urine application, labelled rows display sampling regions and T1 to T6 represent successive sampling time points, where further time points were sampled from parallel mesocosms.

3.2.4 Monitoring nitrous oxide emissions and changes in soil properties

Soil from each sampling zone (see Fig. 3.1; ca. 53 g) was removed, weighed and placed into gas-tight polypropylene containers (total volume 700 cm^3) fitted with a silicone Suba Seal® (VWR International, Lutterworth, UK). Gas samples (20 ml) were taken at 0 and 60 min following container lid closure and were stored in pre-evacuated 20 ml glass vials. The linearity of gas build up within the containers was checked by taking four gas samples (0, 20, 40 and 60 min) on each sampling day in zone A, as this was expected to have the highest emissions. Linearity of gas build-up within containers was met ($R^2 > 0.95$) on 29 and 39% of occasions at 50% and 70% WFPS, respectively. Chadwick et al. (2014) state that non-linear fluxes can arise during occasions of no significant net flux. Our data supports this in that where the linear assumption was violated, the fluxes tended to be minimal and during periods of high emissions the data fitted well to the linear model. We, therefore, consider this acceptable as a poorly fitted linear model at periods of non-significant fluxes is unlikely to cause excessive bias in overall emission estimates. In addition, a high amount of sampled headspace air (during the linearity checks), may have resulted in poorly linear data.

Gas samples were analysed for N_2O with a Varian 450 GC (Agilent Technologies, Santa Clara, CA), fitted with a ^{63}Ni electron capture detector (ECD), where the column, injector and detector temperatures were 50, 100 and 330°C, respectively. After gas sampling, the soil pH, EC and ORP were measured using the methods described previously. Total dissolved organic C, total dissolved N, NH_4^+ and NO_3^- were measured following extraction of the excavated soils with 0.5 M K_2SO_4, as described previously (Section 2.1).

3.2.5 Statistical analysis

All statistical analyses were performed in either SPSS Statistics 20.0 (IBM UK Ltd, Portsmouth, UK) or Minitab 17.1.0 (Minitab Inc., State College, PA). Spatial and temporal

differences between soil pH, EC and ORP in Zones A to E were compared to the control (zone F), and differences between soil incubated at 50% and 70% WFPS were determined via one-way ANOVA, followed by Fisher's LSD post-hoc test. Normality and homogeneity of variance assumptions were tested on log-transformed data using Shapiro Wilk and Levene's test, respectively and significance was determined at the $p < 0.05$ level. Cumulative N_2O emissions were determined by integration using the trapezoidal rule, and differences between the cumulative emissions in each zone were compared via one-way ANOVA, as above. The N_2O emission factor (EF) for each treatment was calculated via the following equation:

$$EF = \text{treatment } N_2O\text{-}N - \text{control } N_2O\text{-}N \,/\, \text{Total N applied} \times 100\% \qquad \text{(Eqn. 3.1)}$$

Differences in emission factors between Zone A and the sum of Zones A-D were compared via one-way ANOVA, as above.

In order to determine the amount of variation in N_2O emissions explained by measured soil parameters, multiple linear regression was used. Data were ln transformed where the distribution was improved by the transformation, in order to approximate normality. During exploratory data analysis, best subset's regression was used; this procedure compares all models for a given set of predictor variables (pH, EC, ORP, Total N, DOC, NO_3^-, NH_4^+, and WFPS), and provides summary statistics (R^2, adjusted R^2, predicted R^2, S and Mallows' Cp) for the best two candidate models with increasing numbers of fixed predictor variables. The number and type of predictor variables were chosen based on the criteria of having a high R^2, adjusted R^2 and predicted R^2, a low value of S (which represents the standard deviation of the error term) and Mallow's Cp values close to the number of terms in the model. The best candidate models were then inputted into the normal multiple regression regime in Minitab.

3.3 Results

3.3.1 Urine patch pH, EC and ORP

Spatial and temporal variation in soil pH, EC and ORP was observed in the Eutric Cambisol following sheep urine application (Fig. 3.2). In Figure 3.2, data are only displayed for the zones A (Fig. 3.2a, e and i), B (Fig. 3.2b, f and j), C (Fig. 3.2c, g and k) and F (Fig. 3.2d, h and l), as data for zones D (see Appendix 1, Fig. 2a, c and e) and E (see Appendix 1, Fig. 2b, d and f) were similar to zone F. The initial soil pH was 6.87 ± 0.15 (50% WFPS) and 6.84 ± 0.16 (70% WFPS; Fig. 3.2d), which increased immediately to pH 8.58 ± 0.08 (50% WFPS) and 8.21 ± 0.14 (70% WFPS) following urine deposition to zone A (Fig. 3.2a). During the first 3 days of incubation, the pH in zone A (50% WFPS) was more alkaline ($p < 0.01$) than that of the control (zone F; Fig. 3.2d). By day 7 the pH had returned to a similar ($p > 0.05$) value to zone F, however, after 10 days the pH was more acidic ($p < 0.01$) than soil previously unaffected by urine, and remained so for the duration of the experiment. Differences in pH, in comparison to the control, only extended to zone B (Fig. 3.2b); this zone was more alkaline ($p < 0.05$) in comparison to zone F (Fig. 3.2d) immediately after urine deposition and returned to the control value faster (after 2 days) than the immediate area of application (Fig. 3.2a). After 4 days, zone B was more acidic ($p < 0.05$) than that of the control for the duration of the experiment. The spatial and temporal changes in pH were generally very similar at 50% and 70% WFPS.

The Eutric Cambisol had an EC of 68.7 ± 6.1 and 74.8 ± 7.2 µS cm^{-1} (50% and 70% WFPS, respectively) without urine application (Fig. 3.2h), which increased to 367 ± 8.5 and 360 ± 30.8 µS cm^{-1} (50% and 70% WFPS, respectively) immediately following urine application (Fig. 3.2e). The EC of Zone A and B (Fig. 3.2e and f, respectively) of the 50% WFPS treatment was greater ($p < 0.001$) for all sample points in comparison to zone F (Fig. 3.2h). A greater EC was also observed in zones C (Fig. 3.2g) and D (see Appendix 1, Fig. 2c),

however, these only became significant ($p < 0.05$) after 4 and 7 days, respectively, indicating a temporal delay in the lateral movement of solutes. No difference ($p < 0.05$) in EC was observed in the 50% WFPS soil from zone E (see Appendix 1, Fig. 2d) in comparison to the control. At both 50% and 70% WFPS in zones A and B (Fig. 3.2e and f, respectively), the EC was immediately higher than the control (Fig. 3.2h), remaining so for the duration of the experiment; the EC in zone C (Fig. 3.2g) was immediately higher than the control (Fig. 3.2h) at 70% WFPS, however, at 50% WFPS it took 4 days for the EC in zone C to be greater than the control. After 10 days, the EC in zone D at 70% WFPS was greater ($p < 0.001$) than the control (Fig. 3.2h), and remained so for the duration of the experiment.

Following application of urine the ORP in zone A at 50% and 70% WFPS (163 ± 14 and 160 ± 27 mV, respectively; Fig. 3.2i) was lower ($p < 0.001$) than zone F (382 ± 6 and 383 ± 6 mV at 50% and 70% WFPS, respectively; Fig. 3.2l) and increased to control levels after 13 days. The ORP in zone B (Fig. 3.2j) at 50% and 70% WFPS (224 ± 28 and 148 ± 21 mV, respectively) was also lower ($p < 0.01$) than zone F (Fig. 3.2l), and increased to that of the control after one week following urine deposition. The ORP was lower ($p < 0.05$) at 50% WFPS in zone A as opposed to the 70% WFPS treatment (Fig. 3.2i), during days 13-48 after urine application.

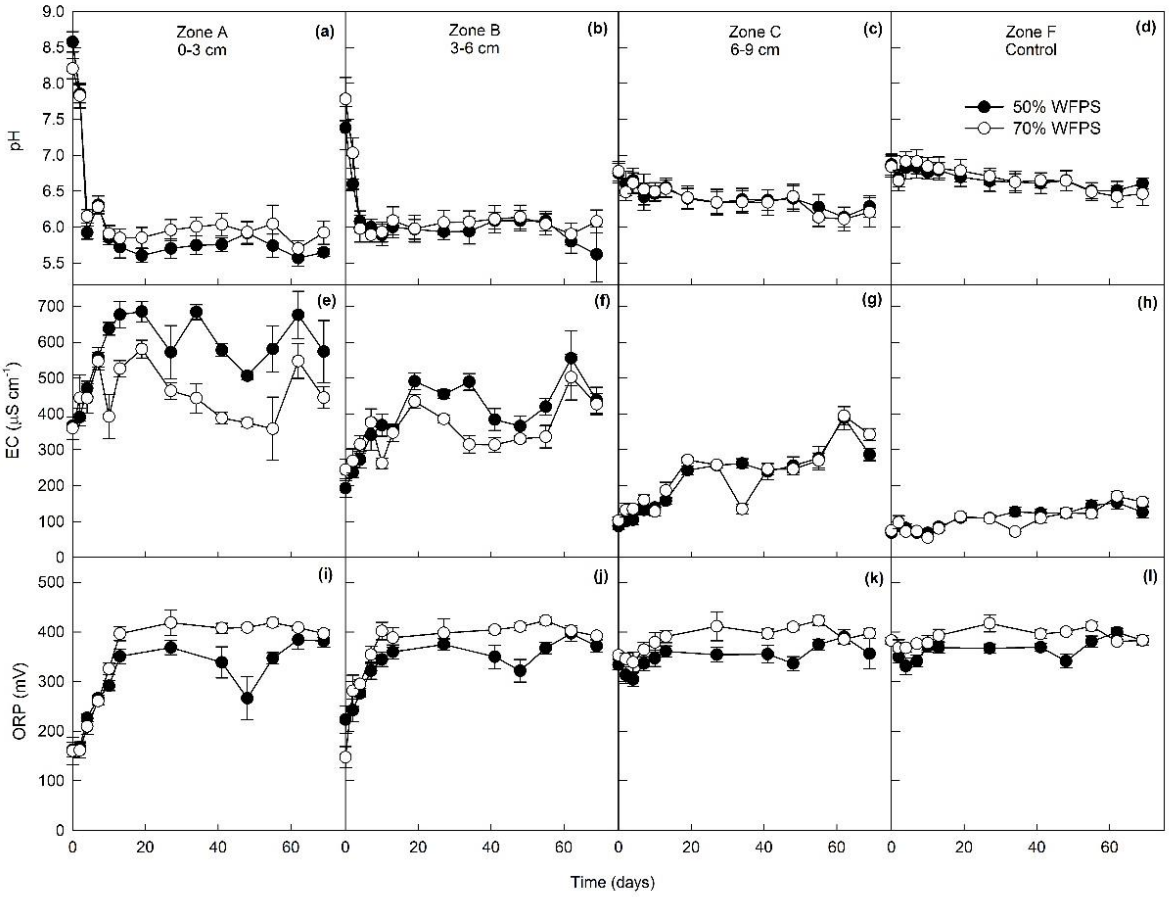

Figure 3.2 Changes in soil pH (panels a, b, c and d), electrical conductivity (EC; panels e, f, g and h) and oxidation reduction potential (ORP; panels i, j, k and l) following sheep urine application to a Eutric Cambisol, maintained at either 50% or 70% water-filled pore space (WFPS), and sampled at increasing distances away from the direct area of application (Zone A, 0-3 cm: panels a, e and i; Zone B, 3-6 cm: panels b, f and j; Zone C, 6-9 cm: panels c, g and k; Zone F, control: panels d, h and l). Symbols represent means ± SEM (n = 4). Figure legend applies to all panels and text on the top row of panels applies to each respective column.

3.3.2 Spatial and temporal dynamics of nitrogen and carbon in the urine patch

Nitrogen and carbon dynamics following sheep urine deposition to a Eutric Cambisol are shown in Figure 3.3, which includes results for total extractable N (Fig. 3.3a, b, c and d),

NH_4^+ (Fig. 3.3e, f, g and h), NO_3^- (Fig. 3.3i, j, k and l), total DOC (Fig. 3.3m, n, o and p) and N_2O emissions (Fig. 3.3q, r, s and t), as no major differences were observed in comparison to the control for Zones D (Appendix 1, Fig. 3a, c, e, g and i) and E (Appendix 1, Fig. 3b, d, f, h and j), only Zones A (Fig 3.3a, e, i, m and q), B (Fig. 3.3b, f, j, n and r), C (Fig. 3.3c, g, k, o and s) and F (Fig. 3.3d, h, l, p and t) are displayed. Most of the applied urine-N was in the form of urea (Table 3.2), which quickly hydrolysed in the soil. This resulted in immediately high soil NH_4^+ concentrations in zone A (50% WFPS; Fig. 3.3e), which peaked at the first sample point at 240 ± 44 mg NH_4^+-N kg^{-1} soil DW. In Zone A of the 70% WFPS soil, the NH_4^+ was high at the first sample point (93 ± 25 mg NH_4^+-N kg^{-1} soil DW) but peaked 3 days following urine application at 140 ± 45 mg NH_4^+-N kg^{-1} soil DW. The NH_4^+ did not diffuse far in the soil and only minor amounts were measured further than zone B.

As nitrification proceeded, the NH_4^+ concentration decreased and a concomitant increase in NO_3^- was observed. In zone A, the NO_3^- concentration peaked 19 days following urine application (Fig. 3.3i), where concentrations were higher in the soil incubated at 50% WFPS (268 ± 9 mg NO_3^--N kg^{-1} soil DW) than the soil incubated at 70% WFPS (207 ± 5 mg NO_3^--N kg^{-1} soil DW). Following the rapid increase in NO_3^- concentration, a decreasing trend was observed over 19–41 days following urine application. After 41 days, the NO_3^- concentration increased at similar rates to that of the control (zone F; Fig. 3.3l) in all zones, but the concentration remained higher than the control in zones A-D. The NO_3^- diffused further than the NH_4^+, and a temporal delay in the diffusion of NO_3^- into outer zones was observed. The major peaks in N_2O emission occurred in zones A and B during the first 20 days following urine application (Fig. 3.3m and n, respectively), while NO_3^- concentrations were still increasing. Emissions peaked immediately following urine deposition, where 882 ± 190 and

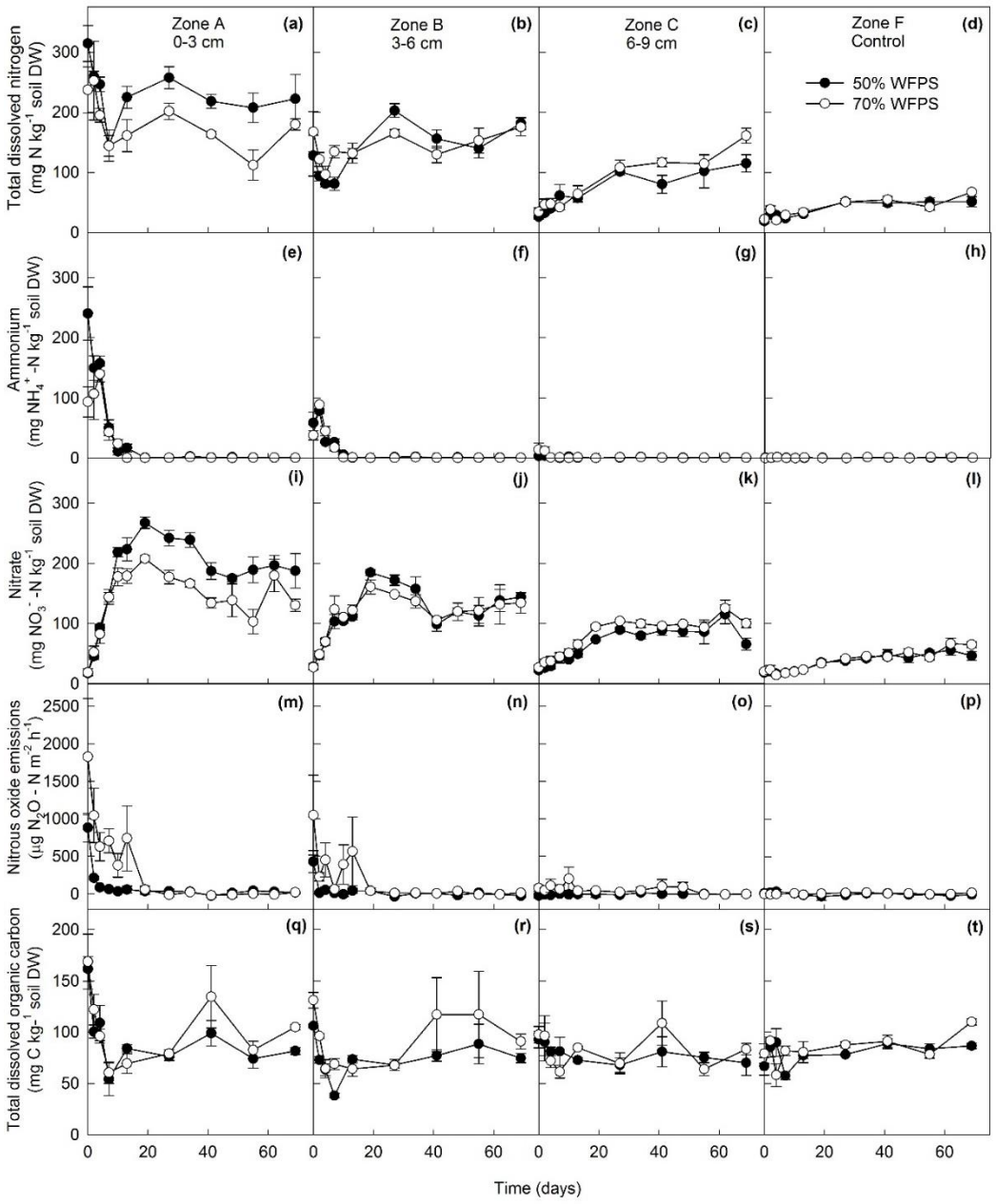

Figure 3.3 Soil extractable total dissolved nitrogen (panels a, b, c, d), ammonium (panels e, f, g, h), nitrate (panels i, j, k and l), nitrous oxide emissions (panels m, n, o and p) and extractable dissolved organic carbon (panels q, r, s and t) following sheep urine application to a Eutric Cambisol, maintained at either 50% or 70% water-filled pore space (WFPS), and sampled at increasing distances away from the direct area of application. Symbols represent means ± SEM (n = 4). Figure legend applies to all panels and text on the top row of panels applies to each respective column.

1825 ± 774 µg N_2O-N m^{-2} h^{-1} were emitted from zone A at 50% and 70% WFPS, respectively. The greatest emissions observed in zone B were also on the day of urine application, and were lower than that of zone A at 431 ± 146 and 1048 ± 531 µg N_2O-N m^{-2} h^{-1} at 50% and 70% WFPS, respectively. Another peak in emissions was observed 13 days following urine deposition in zones A and B, and this was more pronounced in the soil incubated at 70% WFPS. After 20 days following urine application, no major N_2O emissions were measured, yet NO_3^- levels decreased beyond this point.

During the first day of urine application, the concentration of total DOC in the control soil was 66.6 ± 8.6 and 79.1 ± 9.6 mg C kg^{-1} soil DW at 50% and 70% WFPS, respectively (Fig. 3.3t). Due to the presence of labile C within the sheep urine and the potential for urine to solubilise soil organic matter, the concentration of DOC in soil within zone A was 161 ± 12.0 and 169 ± 27.0 mg C kg^{-1} soil DW at 50% and 70% WFPS, respectively (Fig. 3.3q). This rapidly decreased over the course of one week following urine application to 54.0 ± 15.8 and 60.4 ± 10.4 mg C kg^{-1} soil DW in zone A at 50% and 70% WFPS, respectively. A similar trend was observed in zone B (Fig. 3.3r), but at lower initial concentrations (106 ± 1.9 and 131 ± 7.6 mg C kg^{-1} soil DW at 50% and 70% WFPS, respectively) indicating rapid movement and/or solubilisation of DOC into this zone.

3.3.3 Cumulative N_2O emissions and urine patch emission factors

The cumulative N_2O emissions within each zone are displayed in Figure 3.4. Greater cumulative N_2O emissions ($p < 0.01$) were only observed within zone A at 50% WFPS, with respect to the control treatment. At 70% WFPS, both zone A ($p < 0.01$) and zone B ($p < 0.05$) emitted greater amounts of N_2O in comparison to the control. Greater cumulative emissions were observed in the 70% WFPS treatment in comparison to the 50% WFPS in zone A, B and C ($p < 0.01$) but not D, E and F ($p > 0.05$). The emission factor when only considering zone A

was greater ($p < 0.01$) in the soil maintained at 70% WFPS (1.44 ± 0.30% of applied N over 69 days) as opposed to the same soil maintained at 50% WFPS (0.44 ± 0.06% of applied N over 69 days). The N_2O emission factor at 70% WFPS was greater ($p < 0.05$) when summing zones A – D (2.75 ± 0.72% of N applied over 69 days) than when only considering zone A (1.44 ± 0.30% of applied N over 69 days); this was not the case in the 50% WFPS soil, where accounting for the diffusive area had no effect on the N_2O emission factor.

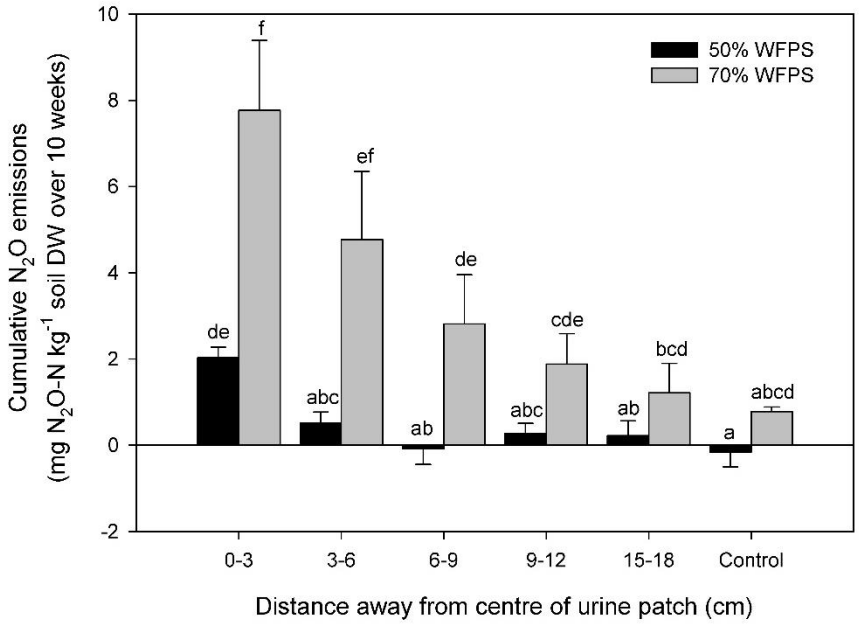

Figure 3.4 Cumulative nitrous oxide emissions following sheep urine application to a Eutric Cambisol, maintained at either 50% or 70% water-filled pore space (WFPS) and sampled at increasing distances away from direct area of urine application. Bars represent means ± SEM ($n = 4$) and different letters indicate significant differences (Fisher's LSD, $p < 0.05$).

3.3.4 Multiple regression analysis

The data included in the multiple regression analysis were those from zones A and B as these regions emitted most N_2O; in addition, the control data (zone F) were also included. The results of the best subset's regression (see Table 3.3) revealed a potential model containing

three predictor variables for individual 'daily' fluxes which fitted the selection criteria well. Increasing the number of variables beyond this did not substantially improve the predictive power of the model, therefore, in order to avoid over-fitting, only three variables were used. The model contained total extractable soil N, ORP and WFPS as predictor variables (R^2: 0.56; adjusted R^2: 0.55, predicted R^2: 0.53; Mallows' Cp: 4.2; S: 0.72). The parameters were then entered separately into the least squares multiple regression model. The results of the regression are presented in Table 3.3 and the regression equations for 50% and 70% WFPS were:

50% WFPS: ln N_2O 'daily' flux = 14.65 – 1.879 ln ORP + 0.2364 ln Total N (Eqn. 3.2)

70% WFPS: ln N_2O 'daily' flux = 15.11 – 1.879 ln ORP + 0.2364 ln Total N (Eqn. 3.3)

Table 3.3 Multiple regression analysis with ln N_2O as the dependent variable, ln total extractable soil nitrogen (TN) and ln oxidation reduction potential (ORP) as predictor variables and water-filled pore space (WFPS; 50% and 70%) as a categorical predictor variable.

Term	**Unstandardized coefficients**		**Standardized coefficients**	***T***	**p**	**VIF**[a]
	B	**SEM**	**Beta**			
(Constant)	5.05	0.09	-	57.0	0.00	-
TN	0.24	0.08	0.19	2.89	0.00*	1.31
ORP	-1.88	0.21	-0.61	-9.09	0.00*	1.32
WFPS (70% vs. 50%)	0.46	0.13	0.21	3.65	0.00*	1.00
Model Summary	***F***	***R²***	***R²* (adj)**[b]	***R²* (pred)**[c]		
	54.6*	0.56	0.55	0.53		

[a] VIF = variance inflation factor, values close to 1 indicate predictors are not correlated.
[b] R^2 (adj) = R^2 adjusted for number of terms in the model.
[c] R^2 (pred) = Predicted R^2, a measure of how well the model predicts the dependent variable for new observations.
* $p < 0.05$

3.4 Discussion

3.4.1 Within-urine patch spatial and temporal variability

The first objective of this study was to determine changes in soil chemical properties of a sheep urine patch both spatially and temporally, and assess how this may influence N_2O production. Within the first few hours of sheep urine application, the soil pH and EC, total extractable N and NH_4^+, N_2O emissions and total DOC had increased while the ORP had decreased in soil directly wetted by the urine. The same trend was observed in zone B, but to a lesser extent, indicating the spread of solutes by mass flow into the region of soil adjacent to the wetted area. The addition of urine to soil may have increased DOC within the soil, due to solubilisation of soil organic matter or lysis of microbial cells by the applied urine (Monaghan and Barraclough, 1993; Ambus et al., 2007; Lambie et al., 2012). The greatest spatial effects were observed for extractable total N and NO_3^-, and EC, whereas all other urine induced soil changes mainly occurred in zones A and B. This is likely to be due to rapid diffusion of NO_3^- and other ions present within ruminant urine through the soil.

In this study the soil pH increased by ca. 2–2.5 pH units, which can be attributed to the high carbonate content of the urine and to alkaline products generated during urea hydrolysis (van Groenigen et al., 2005; Carter, 2007). Soil pH returned to control levels after 7 days, following which it remained more acidic than the control due to the acidifying processes of ammonification, nitrification and urea hydrolysis (Bolan et al., 1991). The spatial changes in pH within a urine patch may be important for within-patch variability and source partitioning of N_2O, as the N_2O product ratios of nitrification, denitrification and dissimilatory NO_3^- reduction to NH_4^+ (DNRA) are all influenced by soil pH (Stevens et al., 1998; Šimek and Cooper, 2002; Mørkved et al., 2007). The pH optimum of nitrification is 6.5-8.0 (Šimek and Cooper, 2002), and these conditions are generally met when ruminant urine is deposited to agricultural soils. In our study the pH dropped below the optimum for nitrification, to values

as low as pH 5.7. Denitrifying enzyme activity has been shown to be highest at, or near, the soils natural pH (Šimek et al., 2002), however, reductions in pH (from 6.82 ± 0.40 to 5.52 ± 0.48) over a 10 month period increased the N_2O/N_2 product ratio of denitrification (Čuhel et al., 2010). In either case, denitrification activity and N_2O release via denitrification are likely to increase once the initial high pH within the urine patch has subsided, whereas N_2O release from nitrification may occur immediately following urine deposition. This may explain the split peak observed in N_2O emissions in this (see Fig. 3.3m and n) and other studies involving urine deposition (e.g. Di and Cameron, 2012; Boon et al., 2014). Alternatively, the second peak may reflect emissions from a more recalcitrant N-containing urine constituent. Advances in the use of stable isotopes, molecular techniques (Bateman and Baggs, 2005; Wrage et al., 2005; Baggs, 2008; Baggs, 2011) and quantum cascade laser based absorption spectroscopy for the measurement of N_2O isotopomers (Waechter et al., 2008; Decock and Six, 2013) will facilitate our understanding of the source partitioning of N_2O following urine deposition to soils.

The differences observed between EC in the different zones revealed a faster lateral movement of solutes at 70% compared to 50% WFPS. This indicates that dilution, mixing and diffusion within soils of a high moisture content may lead to a faster movement of NO_3^- to anaerobic denitrifying microsites within the soil (Luo et al., 1999), where diffusion of soluble carbon may then become limiting (Myrold and Tiedje, 1985). For the majority of the incubation, the soils could be described as moderately oxidized, however, the urine-influenced soil was poorly oxidised at the beginning of the study; this may be due to a localised increase in biological oxygen demand for degradation of the added urinary C (Azam et al., 2002; Baral et al., 2014). Interestingly, the ORP was lower ($p < 0.05$) at 50% WFPS in zone A as opposed to the 70% WFPS treatment, during days 13-48 after urine application. Due to a reduction in the oxygen content of the soils, it may have been expected that the 70% WFPS treatment would have a lower ORP than the 50% WFPS treatment; however, pH and the abundance of oxidizing

and reducing agents can also influence ORP. Here, it is postulated that a greater dilution of oxidizing and reducing agents may have occurred at 70% WFPS, which resulted in a lower ORP at this moisture content. The majority of N_2O emissions occurred when the ORP was between 160 – 350 mV, which is in line with results from studies of paddy and arable soils (Patrick and Jugsujinda, 1992; Yu et al., 2001).

The extractable soil NH_4^+ concentration was initially higher than the control in zone A, indicating rapid urea hydrolysis. The NH_4^+ concentration peaked at 50% WFPS on the day of urine application, however, at 70% WFPS the NH_4^+ concentration peaked 3 days after urine deposition. Increasing moisture increases urease activity up to field capacity, following which it decreases (Dharmakheerthi and Thenabadu, 1996). By using the soil water characteristics estimator of Saxton and Rawls (2006), the WFPS at field capacity was estimated to be 53% which may explain the slight delay in NH_4^+ generation at 70% WFPS as this was above field capacity. The time taken for completion of urea hydrolysis at both moisture contents is similar to that of other studies (e.g. Yadav et al., 1987). As the NH_4^+ was oxidised, the NO_3^- concentration in the urine-influenced soil increased. The major emission period of N_2O took place during the first 20 days after urine application, whilst nitrification was still taking place. As the soils were not completely saturated it is suggested that both nitrification and denitrification contributed to the overall N_2O emissions, due to a combination of aerobic and anaerobic microsites within the soil. Less N_2O emissions at 50% WFPS are consistent with an inhibitory effect of a greater oxygen content upon denitrification. The magnitude of N_2O fluxes were similar to that measured by Allen et al. (1996), where dairy cow urine was applied to pasture blocks in a laboratory incubation.

Minimal N_2O fluxes were observed beyond 20 days of incubation, even though NO_3^- concentrations remained higher than the control, suggesting another factor may have been limiting N_2O production. As temperature and moisture were controlled in this study, it is

suggested that labile C limitation (or DOC being present in a low quality form) prevented N_2O emissions from denitrification, and an NH_4^+ limitation prevented N_2O production via nitrification. Interestingly, NO_3^- concentrations continued to decrease following the major N_2O peak in the absence of plants; possible removal mechanisms are immobilization and diffusion into surrounding soil. Studies investigating the effect of increasing DOC on denitrification rates commonly use glucose as a readily available C source e.g. Weier et al. (1993), however, further work is required to understand how differences in DOC molecular weight and concentration, may influence denitrification rates. Some studies have demonstrated more readily available C compounds stimulate denitrification more than complex molecules (Bremner and Shaw, 1958; deCatanzaro and Beauchamp, 1985) and therefore, determining the effect of DOC species specifically found within ruminant urine on N_2O emissions may explain some of the variability associated with emissions from urine patches related to urine composition.

3.4.2 Predicting N_2O emission by multiple regression analysis

The second objective of this study was to determine the amount of variation in N_2O emissions which could be predicted by the measured soil parameters. In the final multiple regression model changes in total extractable soil N, WFPS and ORP all contributed significantly ($p < 0.001$) to the variation in N_2O emissions, explaining 55.6% of the total variation. Provided all other variables are held constant, increasing the total N by 1% resulted in a 0.24% increase in N_2O emissions and decreasing ORP by 1% resulted in a 1.88% increase in N_2O emissions, under these experimental conditions. N_2O emissions were, on average, 58% higher in soil incubated at 70% in comparison to 50% WFPS, when holding ORP and total extractable N constant. Model parameters which contributed the most new information to the model followed the sequence ORP > WFPS > total extractable N. Low amounts of organic N were extracted from soils following urea hydrolysis and, therefore, the inclusion of total

extractable N in the best subset's regression, as opposed to individual NO_3^- or NH_4^+ concentrations, supports the tenet that the majority of N_2O emissions were due to a combination of nitrification and denitrification.

3.4.3 The importance of urine patch edge effects

The third objective of this study was to determine how important considering urine patch edge effects are when calculating N_2O emission factors. The marked difference between the lateral distribution of NH_4^+ and NO_3^- within the urine patch highlights the importance of considering the urine patch diffusional area when monitoring N_2O emissions via chambers, or studies monitoring N loss via lysimeters. Under field conditions the mass of mineral N available for lateral diffusion may be influenced by plant uptake, the extent of vertical diffusion, leaching and preferential flow through soil macropores. The mesocosms used in this study used homogenised soil (i.e. preferential flow unlikely) and did not include effects of plant uptake, leaching, drainage and vertical diffusion beyond 5 cm. It would be expected that accounting for these processes would result in a lower mass of mineral N available for lateral diffusion than observed in this study. Nevertheless, the lateral diffusion of N in our study was 11 cm less than that observed by Decau et al. (2003), where cattle urine (3 l over 0.4 m^2) was applied to 1 m deep lysimeters with a cross-sectional area of 2 m^2. However, the experimental conditions in Decau et al. (2003) were not similar to those used in this study.

The NH_4^+ derived from the urine application remained central to the urine patch, with only small amounts diffusing up to 3 cm away from the initial wetted area. Conversely, the highly mobile NO_3^- diffused ca. three times as far from the centre of the urine patch and persisted in the soil for a longer period. These results suggest that N_2O production via nitrification would be limited by the lateral diffusion of NH_4^+, and are therefore only likely to occur in the initial wetted area and the area of soil influenced by mass flow of urine through

soil immediately after deposition. On the other hand, denitrification of urinary nitrogen may occur both centrally and within a larger diffusional area of soil around the urine patch.

This suggests that in order for mitigation strategies such as synthetic or biological nitrification inhibitors to be effective at reducing N_2O emissions, it would be beneficial for the inhibitors to possess a similar charge and diffusion coefficient to NH_4^+. As roots can undergo death and decomposition in the direct urine deposition zone (Shand et al., 2002), it is likely that the biological (i.e. plant) delivery of nitrification inhibitors will be of most significance in the diffusive zone. Research regarding biological denitrification inhibition is still in its infancy (Bardon et al., 2014) and further research is required, yet, an effective denitrification inhibitor would ideally match the diffusive speed of NO_3^-.

In this study, emissions were ca. 1.5 and 2 (50% and 70% WFPS, respectively) times greater when considering the wetted and diffusional area (sum of zones A-D) in comparison to the wetted area only (zone A). Under field conditions this figure may be expected to be lower due to some removal of NO_3^- via plant uptake, draining, leaching and vertical diffusion however, it may be more representative of times where plant uptake is low or urine is deposited to areas of bare soil in the field. The walls of chambers for measuring gaseous emissions from soil are generally inserted to a depth of 5 cm. If a urine patch is applied uniformly throughout a chamber, then the chamber walls may prevent lateral diffusion of NO_3^- and DOC into the surrounding soil, resulting in greater concentrations than would have been present otherwise or a deeper infiltration of urine. This could potentially overestimate denitrification, however, due to the limited diffusive speed of NH_4^+ perturbation to N_2O emissions from nitrification may be minimal. On the other hand, not considering the urine patch diffusional area may underestimate N_2O emissions, due to the smaller zone of soil influenced by the urine, resulting in fewer microbes exposed to the addition of N, DOC and moisture. Similarly, due to fewer microbes at soil depth, a greater vertical movement of urine may reduce direct emissions. It

cannot be excluded that the opposing effects could cancel each other out. Further work is required to assess these potential processes, which could be investigated via the use of ^{15}N-labelled urine applied to larger and deeper pasture mesocosms with an intact sward, comparing source-partitioned emissions with and without a chamber wall to restrict diffusion.

3.4.4 Theoretical diffusion of NO_3^- and NH_4^+ through soil

A calculation of the theoretical diffusive speed of NO_3^- and NH_4^+ through soil may be useful for estimating the urine patch diffusional area in differing soils (and hence the additional area required within chambers to improve accuracy of emission measurements). To assess this we compared the theoretical linear distance of diffusive movement to the observed diffusive movement in the mesocosms. The effective diffusion coefficient (D_e) can be calculated using the equation

$$De = \frac{D_1 \times \theta f \times dC_1}{dC_s} \qquad \text{(Eqn. 3.4)}$$

where D_1 is the diffusion coefficient in pure water, θ is the soil volumetric moisture content, f is the impedance or pore tortuosity factor and dC_1/dC_s is the reciprocal of the buffer power (Nye and Tinker, 2000). To calculate D_e in the mesocosms we used D_1 values of 1.60 and 1.64 $cm^2\ d^{-1}$ for NO_3^- and NH_4^+, respectively (Lide, 2004), an f value of 0.3 (Jones et al., 2005) and the moisture contents of the mesocosms. Values for the buffer power in the same soil, were obtained from (Jones et al., 2012). Further, the linear distance (L) of diffusive movement of NO_3^- and NH_4^+ through time can be calculated as

$$L = (2D_e t)^{1/2} \qquad \text{(Eqn. 3.5)}$$

where *t* is time. Using these parameters, the linear diffusive distance of NH_4^+ over 10 weeks was calculated as 1.50 and 1.72 cm at 50% and 70% WFPS, respectively. The calculated diffusive distance of NO_3^- was greater at 4.18 and 4.94 cm at 50% and 70% WFPS, respectively. In the soil mesocosms, increased NH_4^+ was observed up to 3 cm from the urine patch edge, whereas increased NO_3^- concentrations were observed up to 9 cm from the urine patch edge. Some disparity between observed and theoretical values may be due to the coarser scale of measurement (3 cm fractions) in the mesocosms and saturation of the exchange phase with urine derived K^+ (and other ions) which could lower the sorption of NH_4^+. The formation and subsequent diffusion of NO_3^- may have occurred after the NH_4^+ had diffused 1.50–1.72 cm, which may be the reason for the greater observed compared to theoretical diffusive distance of NO_3^-. Further validation of this method by comparison to measured urine patch diffusional areas in the field, across varying soil types, soil moisture contents, microtopography and urine patch N concentrations and volume need to be investigated prior to utilising this equation as a method for determining the chamber size required for an experimental urine patch.

3.5 Conclusions

The results of our study show that N_2O emissions can extend beyond the initial wetted area of a urine patch, and that this effect is greater under a high soil moisture content. For a typical sheep urine application to a Eutric Cambisol with an even surface, an additional 9 cm around the initial wetted area would have been required to capture the majority of N_2O emissions via a chamber based system. The additional area required around a urine patch may also vary alongside urine volume, patch area, the concentration of N applied, the soil type beneath the patch and the underlying microtopography. These conditions are likely to be highly site specific, therefore, preliminary assessments should be conducted in order to assess the

magnitude of the urine patch diffusional area, and additional area inside chambers should be allowed for, prior to monitoring emissions.

3.6 Acknowledgments

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3.7 References

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Chapter 4

Disentangling the effect of sheep urine patch size and N loading rate on cumulative N_2O emissions

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KAM, DLJ and DRC designed and conceived the experiment; KAM conducted the experimental work, analysed the data and prepared the manuscript.

Abstract

Ruminant urine N concentration and volume are important parameters influencing the size and N loading rate of urine patches deposited to soil. Such parameters can influence N cycling and emissions of the greenhouse gas, nitrous oxide (N_2O) from grazed grassland, yet, there is limited information on the effect of these parameters within typical ranges reported for sheep. We used an automated, high frequency gas monitoring system to investigate N_2O emissions from varying urine N application rates and patch sizes under field conditions. Using artificial sheep urine, we manipulated urine N concentration to provide two urine N application rates (4 and 16 g N l^{-1}; equivalent to 200 and 800 kg N ha^{-1}). We investigated the effect of urine patch size with equal N application rates (4 × 125 cm^2 vs. 500 cm^2, at 200 and 800 kg N ha^{-1}) and the effect of patch size with unequal N application rates, but the same total amount of N applied (62.5 ml over 125 cm^2 at 800 kg N ha^{-1} and 250 ml over 500 cm^2 at 200 kg N ha^{-1}). Cumulative emissions of N_2O generally increased with N loading rate, whether applied as one large urine patch or four smaller ones. Cumulative N_2O emissions increased when the N was applied in four smaller urine patches compared to one large patch; this difference was significant at 800 kg N ha^{-1}, but not at 200 kg N ha^{-1}. When the total amount of N applied was held constant (1 g of N), the amount of N_2O released was similar when urine was applied as a high N concentration small patch (800 kg N ha^{-1}) compared to a low N concentration large patch (200 kg N ha^{-1}). Urine N_2O emission factors in this study were, on average, ten times lower than the IPCC default of 1% for sheep excreta. This research clearly demonstrates that the chemical and physical nature of the urine patch influences N_2O emissions, yet further research is required to gather more data on typical sheep urine volumes (individual and daily), urination frequency, urine N concentrations and the typical volumes of soil influenced by urine deposition, to provide more accurate estimates of emissions from sheep grazed pastures.

Key Words: agricultural systems; global climate change; microbial processes; ruminants.

4.1 Introduction

Ruminant urine N concentration, volume and frequency vary widely, influencing the size and N loading rate of urine patches deposited to pasture soils. These parameters can influence the fate of urinary N in grazed pastures, including emissions of the powerful greenhouse gas nitrous oxide (N_2O) and leaching of NO_3^- (Li et al., 2012). The default IPPC N_2O emission factor for both cattle and sheep was 2% of applied N, however, due to the lower volume and higher frequency of sheep urine events, and more moderate levels of soil compaction under sheep-grazed pastures, the default emission factor has been lowered for sheep from 2% to 1% of the applied N (IPCC, 2006). The range in urine volumes deposited by sheep and cattle (0.13-2 l) has been shown to influence N transformations and processes (e.g. longer retention of mineral N, greater plant growth and N uptake in larger patches) even when the total N applied is constant (Orwin et al., 2009). Whilst these results display a clear difference between sheep and cattle urine patches, where large differences in urinary volume are produced between the two species, less work has been conducted on N cycling and N_2O emissions within the typical ranges of urine patch sizes reported for sheep.

The concentration of N excreted in urine is a function of the amount of surplus metabolized N to be excreted, the volume of urine produced and the frequency of urine events (Hoogendoorn et al., 2010) and can range from 1-18 g N l^{-1} (Bristow et al., 1992; Oenema et al., 1997; Hoogendoorn et al., 2010). Urine volume is mainly influenced by water intake and the mineral load ingested by the animal (Selbie et al., 2015), and can be high when the moisture content of the diet is high, or when the herbage leaves are wet with rain water or dew (Doak, 1952). Urine volume can also vary as a response to coping with changes in ambient temperatures (Betteridge et al., 2010a). Data for sheep urine volume and frequency are scarce, but typically individual sheep urine events range between 100-200 ml (Doak, 1952; Haynes and Williams, 1993). However, variation within individual sheep urine volumes can be high

e.g. the mean and range of 40 individual urine events between 6 Welsh Mountain ewes fed (*ad libitum*) a *Lolium perenne* L. dominated sward was 104 (18-397) ml (Marsden, unpubl.). Daily sheep urine volume ranged from 0.5-3 l in Ledgard et al. (2008) and the frequency of urination events is reported to range between 13-20 times per day (Doak, 1952; Betteridge et al. 2010a; Betteridge et al., 2010b).

Urine volume, soil moisture status, soil type and topography can all influence the urine patch wetted area and volume of soil influenced by a urine patch. The wetted area of a urine patch has been defined as the surface area covered by urine following deposition to soil, as opposed to the pasture response area which can extend beyond the wetted area (Lantinga et al., 1987; Li et al., 2012). Typical wetted areas for a sheep urine event with a volume of 150 ml is reported to be 300 cm^2 (Doak, 1952) and for 200 ml is reported to be 430-550 cm^2 (Williams and Haynes, 1994). Typical urine applications of 4 l m^{-2} were reported to be utilised in Kelliher et al. (2014).

Due to the wide range in sheep urine volume and frequency, and the interactive effects of dietary N content, energy use, and ambient temperature fluctuations it is evident that sheep urine patches vary widely in N concentration and volume, resulting in patches of different sizes and of different N loading rates. It can be envisaged that at times where sheep may be fed a diet low in moisture content, or when ambient temperatures are high, that their urine will be more concentrated, having a higher N concentration deposited over a small patch size. On the other hand, when the diet is high in moisture and the sheep is fully hydrated, urine may be deposited in larger sized urine patches with a lower N concentration. This experiment was designed to test these two extremes, to determine how differences in sheep urine patch size and N concentration may influence N cycling and cumulative N_2O emissions arising from such urine patches.

Using a high frequency automated greenhouse gas monitoring system we assessed whether: 1) cumulative N_2O emissions will increase with increasing urine N loading rate, when keeping the urine patch size constant, 2) cumulative emissions will be higher from four small urine patches compared to one large urine patch, when keeping the total N loading rate constant, and 3) cumulative emissions will be higher from a low N concentration large urine patch compared to high N concentration small urine patch, where N loading rate differs, but the total amount of N applied remains constant. The total amount of applied N equates to the total amount of N in the volume of applied urine. The N loading rate is a function of the urine N concentration and the surface area the urine is applied to (Selbie et al., 2015). We also examined the effect of reduced sampling frequency (i.e. reflecting the typical static chamber measurement approach) on measured cumulative N_2O emissions.

4.2 Materials and methods

4.2.1 Field site

The field site was established at Henfaes Research Station, Abergwyngregyn, North Wales (53°14′N, 4°01′W) in March, 2015. The soil at the site is classified as a Eutric Cambisol and is of mixed glacial till in origin, deposited ca. 10,000 years ago. The field was reseeded in 1990 with a *Lolium perenne* L. and *Trifolium repens* L. mix. The field has since received moderate fertilizer applications (ca. 120 kg N ha^{-1} y^{-1} in years 1990-2002 and ca. 60-80 kg N ha^{-1} y^{-1} in subsequent years) and is regularly grazed by Welsh Mountain ewes at a moderate stocking density (2-3 livestock units / ha). The area was fenced off just over 3 months prior to treatment application, to prevent the effect of recent livestock urine patches on monitored gas fluxes from the soil.

4.2.2 Soil sampling and analysis

Soil (0-10 cm) was sampled in triplicate at block level (n = 3) to determine soil characteristics (Table 4.1); this was conducted four days prior to treatment application. Soil moisture content was determined by oven drying (105ºC; 24 h) and organic matter was determined by the loss-on-ignition (450 ºC; 16 h; Ball, 1964). Soil pH and electrical conductivity (EC) were determined using standard electrodes in 1:2.5 (w/v) soil-to-distilled water suspensions. Total C and N were determined on oven-dried and ground samples in a TruSpec® Analyzer (Leco Corp., St. Joseph, MI). Dissolved C and dissolved N were determined in 1:5 (w/v) soil-to-0.5 M K_2SO_4 extracts, according to Jones and Willet (2006). Microbial biomass C and N were determined via the chloroform fumigation-extraction method of Voroney et al. (2008), using K_{EC} and K_{EN} correction factors of 0.35 and 0.5, respectively. Total extractable P, NO_3^-, and NH_4^+ were determined in 0.5 M K_2SO_4 extracts via the methods of Murphy and Riley (1962), Miranda et al. (2001) and Mulvaney (1996), respectively. Exchangeable cations (Na, K, Ca) within 1:5 (w/v) soil-to-1 M NH_4Cl extracts were measured using a model 410 flame photometer (Sherwood Scientific Ltd., Cambridge, UK).

4.2.3 Experimental design and treatment application

Artificial sheep urine was made up according to Lucas and Jones (2006), the composition of which is in line with the suggestion of Kool et al. (2006) in containing at least urea and hippuric acid for a realistic simulation of N_2O emissions from artificial urine patches. The N concentration of the artificial urine was modified by adjusting the proportion of urea to obtain a total of 4 g N l^{-1} and 16 g N l^{-1}, where all other urine constituents were held constant. The two N concentrations were chosen to reflect low and high N concentrations in the urine, within typical reported ranges for N concentration within sheep urine. The effect of urine patch size was investigated by comparing a single large urine patch with four smaller patches, while

holding total N loading rate constant. The effect of patch size with unequal N loading rates, but the same total amount of applied N, was also assessed, by comparing a low N concentration large urine patch to a high N concentration small urine patch.

Table 4.1 Properties of the Eutric Cambisol at the field site receiving artificial urine application. Values represent means ± SEM (n = 3) and results are reported on a dry weight basis.

Eutric Cambisol properties	
Texture	Sandy Clay Loam
Bulk density (g cm^{-3})	1.08 ± 0.05
Gravimetric moisture content (%)	42.8 ± 0.80
Organic matter (%)	11.1 ± 0.68
pH	6.08 ± 0.04
EC (µS cm^{-1})	41.7 ± 8.1
Total C (%)	5.47 ± 0.77
Total N (%)	0.42 ± 0.05
C:N ratio	12.8 ± 0.65
Dissolved organic C (mg C kg^{-1})	109 ± 13.7
Total dissolved N (mg N kg^{-1})	16.2 ± 0.82
Microbial biomass C (g C kg^{-1})	2.32 ± 0.09
Microbial biomass N (mg N kg^{-1})	186 ± 2.60
Extractable NO_3^- (mg N kg^{-1})	0.05 ± 0.01
Extractable NH_4^+ (mg N kg^{-1})	1.47 ± 0.15
Extractable P (mg P kg^{-1})	6.56 ± 1.21
Exchangeable Na (mg kg^{-1})	13.4 ± 1.74
Exchangeable K (mg kg^{-1})	123 ± 37.5
Exchangeable Ca (g kg^{-1})	0.46 ± 0.07

The study was set up in a randomised block design, with 5 treatments (n = 3), as shown in Appendix 2 (Fig. 1). The treatments were as follows: 1) control (no urine application), 2) large urine patch with a low N concentration (4 g N l^{-1}; 1 g of N applied in 250 ml covering 500 cm^2; N loading rate = 200 kg N ha^{-1}), 3) large urine patch with a high N concentration (16 g N l^{-1}; 4 g of N applied in 250 ml, covering 500 cm^2; N loading rate = 800 kg N ha^{-1}), 4) four

small urine patches with a low N concentration (4 g N l^{-1}; 4 × 0.25 g of N applied in 4 × 62.5 ml, covering 125 cm^2 each; N loading rate = 200 kg N ha^{-1}) and 5) four small urine patches with a high N concentration (16 g N l^{-1}; 4 × 1 g of N applied in 4 × 62.5 ml, covering 125 cm^2 each; N loading rate = 800 kg N ha^{-1}).

Duplicate plots were established, one to allow for chamber gas flux measurements and the other for soil sampling during the study. Artificial urine was applied by evenly pouring onto the soil, using a fixed template as an area guide. All urine patches were smaller than the chamber basal area (2500 cm^2), ensuring additional room inside the chamber for the urine patch diffusional area, which is important for accurately monitoring urine patch N_2O emissions (Marsden et al., 2016). Soil cores (0-5 cm) were taken from the area of immediate urine application, during the course of the study. Soil sampling was conducted four days prior to treatment application (for measurement of background soil characteristics), on the day of a urine application, and 2, 4, 7, 10, 14, 17, 21, 28 and 35 days following treatment application. The soil sampling was conducted more frequently at the beginning of the study (three times in the first week) in order to provide information on soil N transformation processes which occur relatively quickly following organic N additions to soil (e.g. urea hydrolysis and nitrification). Soil sampling reduced in frequency throughout the remainder of the study (twice per week in the second and third week, and once per week, thereafter). When soil cores were taken, they were timed to match manual flux measurements taken from the control plots. Soil cores were returned immediately to the laboratory, where post processing took place within 24 h of sampling. Prior to extraction, soils were homogenised by gently mixing within the sample bags.

Soil moisture was monitored within each individual chamber by using Acclima SDI-12 digital TDT® sensors (Acclima Inc., ID, USA), monitoring at a 0.5 h measurement frequency. The sensors were inserted diagonally through the urine patch, and were in situ 3 weeks prior to urine application. Rhizon suction samplers (2.5 mm diameter, 5 cm porous tube length, 12 cm

tubing length; Rhizosphere Research Products, Wageningen, Netherlands) were also inserted at an angle of 45º, into the centre of individual urine patches, 3 weeks prior to urine application. This allowed sampling of soil solution within the chamber in a non-destructive manner when the soil was wet enough for sample collection. Successful soil solution samples were collected 4 and 3 days prior to urine application, on the day of urine application and 2, 4, 7, 10, 14 and 17 days following urine application. Beyond this point soil conditions were too dry, so samples were not collected. Additionally, air temperature was monitored both inside and outside chambers using Thermochron iButtons® (iButtonLink, LLC, WI, USA) logging temperature every 1 h. A weather station was located near to the field site, where rainfall, soil (0-10 cm) and air temperature were monitored hourly.

4.2.4 Pasture biomass and N content

The pasture within chambers was cut 2 and 4 weeks following urine application, to a height of 5 cm. Samples were oven dried (80ºC; 24 h), weighed for biomass, and ground before analysis of N content as described previously.

4.2.5 Automated and manual greenhouse gas measurements

Greenhouse gas fluxes were measured from twelve non-steady-state, non-through flow chambers connected to a mobile, automated high frequency measurement system (Queensland University of Technology, Institute for Future Environments, Brisbane, Australia) as detailed in Scheer et al. (2014). Stainless steel chamber bases were inserted into the ground (10 cm depth) for 4 weeks prior to treatment application, to ensure no effects from soil disturbance were observed during the study. Chambers (50 cm x 50 cm x 15 cm) were fixed to the bases, which opened and closed during sampling via pneumatic actuators. Chamber headspace samples were automatically pumped (ca. 200 ml min^{-1}) from the chambers, through Teflon

tubing to the sampling unit, which housed a LI-COR LI-820 non-dispersive infrared gas analyser (LI-COR, St Joseph, MI, USA) to measure the CO_2 concentration, and a gas chromatograph (SRI 8610C, Torrance, USA), equipped with a ^{63}Ni electron capture detector (ECD) and flame ionization detector (FID) to measure N_2O and CH_4 concentrations in the chamber headspace, respectively. The samples passed through an Ascarite (sodium hydroxide coated silica) column prior to entering the GC, in order to remove moisture and CO_2, which was changed periodically in order to protect the ECD cell.

The sampling routine consisted of three blocks of four chambers, where each block of chambers close sequentially for a period of 1 h, while the other two sets are open allowing restoration of ambient conditions. During the 1 h closure period each chamber is sampled for 3 min, followed by a calibration standard (500 ppb N_2O; 880 ppm CO_2; 3 ppm CH_4; ± 2 % of the certified value, BOC gases, Liverpool, UK). The 15 min cycle repeats four times, before the next set of chambers are sampled. One entire cycle takes 3 h to complete, allowing up to 8 flux measurements per 24 h period.

The automated system consisted of twelve chambers only, therefore, control treatments were sampled manually from static chambers (n = 3), as N_2O fluxes were expected to be minimal from this treatment. Manual chamber flux measurements were taken once per day, between 10 am and 12 noon (de Klein and Harvey, 2012) as close to daily as possible, for the duration of the experiment. The gas samples were taken by placing polypropylene upturned buckets (ca. 26 cm in height) onto collars (26 cm diameter), inserted to a depth of 10 cm. The chambers were fitted with a re-sealable vent, to allow pressure equalisation when placing chambers onto bases, and were fitted with Suba-Seals ® (Sigma, Gillingham, UK) to allow sampling of the headspace. Samples were taken with a syringe every 15 min, over the period of 1 h, to match the automated system. Samples were stored in pre evacuated 20 ml glass vials,

before being analysed on a Clarus 500 GC with a TurboMatrix headspace autoanalyser (Perkin Elmer, CT, USA).

4.2.6 Statistical analysis

Cumulative N_2O emissions were determined by integration using the trapezoidal rule. The resulting cumulative emissions were corrected for the chamber area assumed to be unaffected by urine application (i.e. total chamber area = 0.25 m^2, urine patch area = 0.05 m^2 and control area unaffected by urine = 0.2 m^2), by deducting the cumulative emissions arising from the control treatment over an area of 0.2 m^2. Further calculations e.g. emission factors were based on the control-corrected values for cumulative emissions. To compare whether cumulative emissions or emission factors increased with increasing N loading rate, t-tests were conducted, comparing the results from treatments 2 with 3 and treatment 4 with treatment 5. In order to determine whether emissions are greater from small or large urine patches (with the same N loading rate) the same procedure was used, comparing treatment 2 with treatment 4 and treatment 3 with treatment 5. To test the third hypothesis that cumulative emissions would be higher from a low N concentration large urine patch (200 kg N ha^{-1}) compared with a high N concentration small urine patch (800 kg N ha^{-1}) with the same total amount of N applied, the results from treatment 5 were divided by 4 (assuming that cumulative emissions from each individual urine patch within the chamber were equal) and compared with the results from treatment 2. Differences in pasture biomass and foliar N content at 2 and 4 weeks following urine application were assessed via ANOVA with Tukey's post-hoc test, after assessing normality and homogeneity of variance.

To assess how the accuracy of cumulative N_2O emissions from urine patches is affected by the frequency of sampling, we compared the high frequency N_2O dataset with the N_2O dataset containing (where available): 1) daily measurements between 10 a.m. and 2 p.m., 2) weekly measurements between 10 a.m. and 2 p.m., and 3) a sampling campaign where 3 flux

measurements were taken in the first week after urine application, 2 flux measurements in the second week after urine application and once a week thereafter. These different (infrequent sampling) regimes reflected those typically used in the literature for determining emissions from urine patches. Differences were reported as the mean across all replicates and treatments. All statistical analysis was conducted in Minitab 17.0 (Minitab Inc., State College, PA, USA).

4.3 Results

4.3.1 Effect of urine N content and patch size on soil C and N dynamics

The dynamics of total (free and exchangeable) NH_4^+ and NO_3^- concentrations in soil can be seen in Fig. 4.1a and b, respectively, while the concentration of NH_4^+ and NO_3^- in soil solution (extracted with Rhizon samplers within the chambers) is presented in Fig. 4.1c and d. During the second half of the field trial the lack of rainfall prevented the acquisition of soil water with the Rhizon samplers and thus, there are no results to present. Extractable NH_4^+ increased up to 4 days following urine application in the treatments receiving a high N loading rate, and then followed a declining trend. As expected, extractable NH_4^+ peaked at a lower concentration in the lower N containing treatments, and began to decline two days following urine application. There were no apparent differences in the urine-induced increase of soil NH_4^+ concentration due to patch size. The NO_3^- concentration steadily increased in the soil extracts and soil solution, with lower amounts of NO_3^- produced in the low N containing treatments. Differences in NO_3^- concentration between patch sizes were evident, with less accumulation of NO_3^- in smaller patches, and with concentrations returning to background values faster than in large urine patches. In the high N containing large urine patch, the NO_3^- concentration was still higher than the control at the end of the experiment, and it cannot be ruled out that further N_2O emissions would have occurred after this time.

The total extractable dissolved N and organic C present in soil during the course of the study can be seen in Fig. 4.2a and b, respectively, while the soil solution total dissolved N and C can be seen in Fig. 4.2c and d, respectively. Concentrations of extractable and soil solution

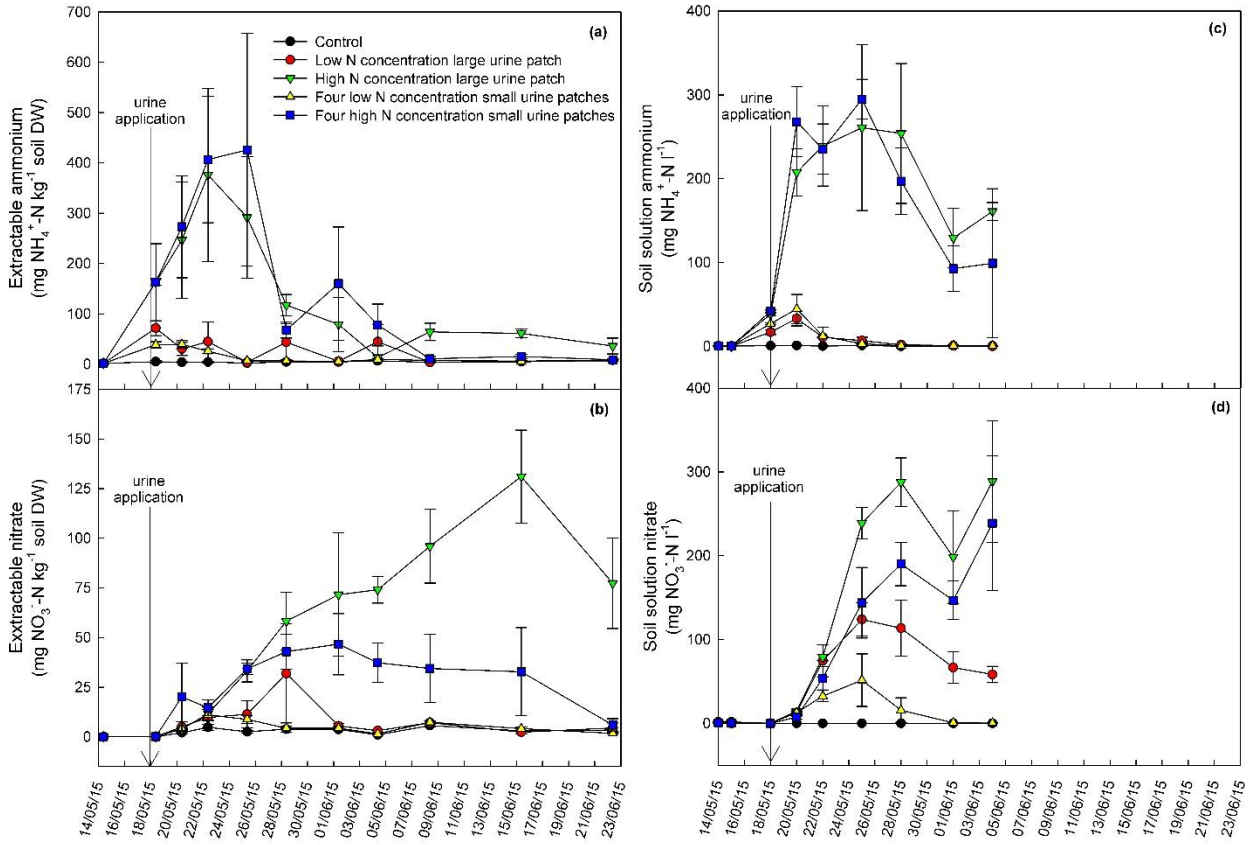

Figure 4.1 Mineral N dynamics following artificial sheep urine application of varying N concentration and patch size to a Eutric Cambisol, panel a) extractable soil ammonium-N panel b) extractable soil nitrate-N, panel c) soil solution nitrate-N and panel d) soil solution ammonium-N. Figure legend applies to all panels, symbols and error bars denote means ± SEM, where *n* = 3 for panels a) and b), however, *n* varies (1-3) for panels c) and d), due to variability in successful soil solution collection by Rhizon samplers.

N were high at the beginning of the study, due to the large amounts of dissolved organic N deposited into the soil within the urine. The N persisted in the soil for longer periods in the high N containing urine patches. The soil solution C was immediately high following urine application, and rapidly declined to control values. Dissolved organic C may have been higher

in the high N containing treatments, due to the increased proportion of urea in these treatments, and/or it may have been caused by a priming effect, which has been demonstrated for ruminant urine-influenced soils for both C (Lambie et al., 2013) and N (Di and Cameron, 2008).

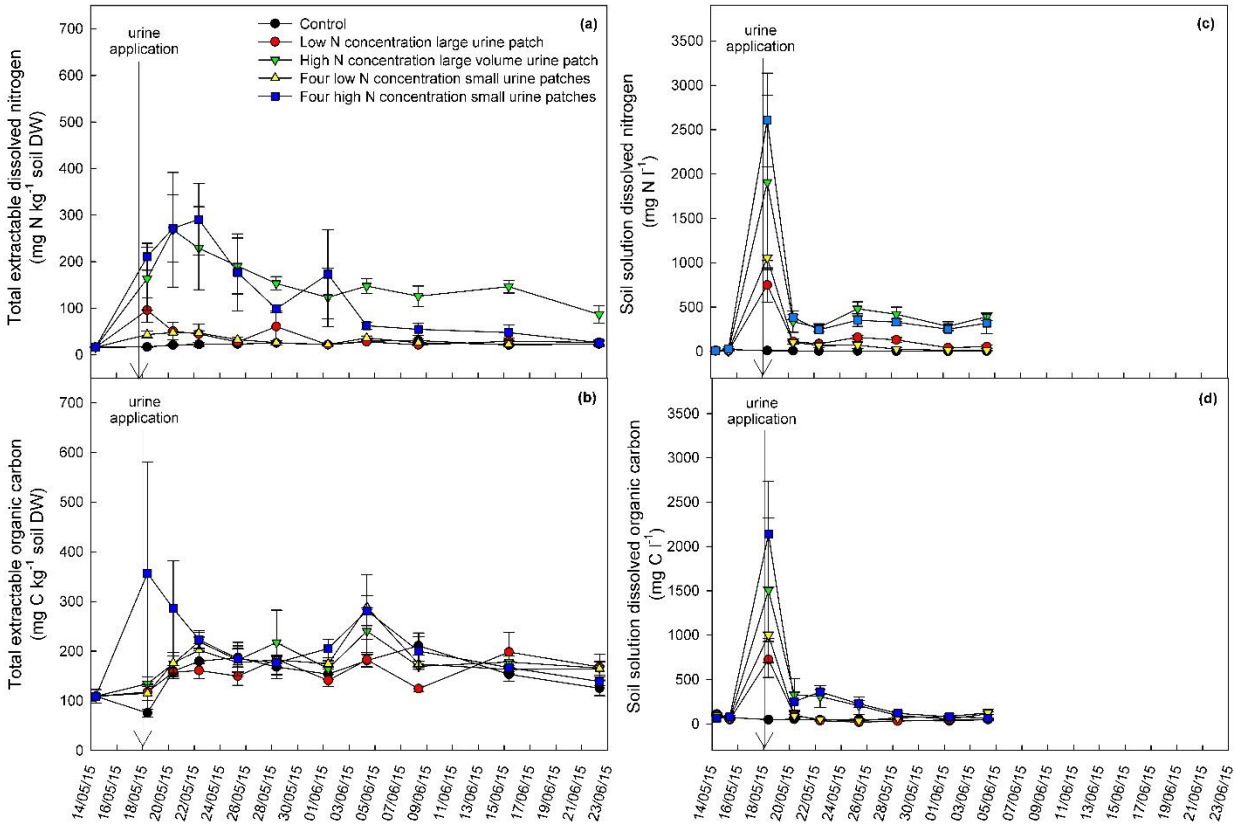

Figure 4.2 Dynamics of total extractable nitrogen and organic carbon (panels a and b, respectively) and soil solution nitrogen and dissolved organic carbon (panels c and d, respectively) following artificial sheep urine application (of varying patch size and N content) to a Eutric Cambisol. Figure legend applies to all panels, symbols and error bars denote means ± SEM, where *n* = 3 for panels a) and b), however, *n* varies (1-3) for panels c) and d), due to variability in successful soil solution collection by Rhizon samplers.

4.3.2 Pasture biomass and foliar N content

The pasture biomass and N content after 2 and 4 weeks following urine application can be seen in Table 4.2. As expected, the pasture biomass and foliar N content were generally larger in the urine treatments compared to the control (except pasture biomass 4 weeks after urine application). Increasing the N loading rate in a single large urine patch did not increase ($p > 0.05$) pasture biomass 2 or 4 weeks after urine application, nor did it increase foliage N content in the first 2 weeks after urine application, however, foliage N did increase after 4 weeks ($p < 0.05$).

Table 4.2 Pasture biomass (g DM m^{-2}) and foliar N content (%) at 2 and 4 weeks following artificial sheep urine application to a Eutric Cambisol. Values represent means ± SEM (n = 3), and letters indicate significant differences ($p < 0.05$).

	2 weeks after urine application		4 weeks after urine application	
Treatment	Biomass (t DM ha^{-1})	Foliar N content (%)	Biomass (t DM ha^{-1})	Foliar N content (%)
Control	43.7 ± 3.8 a	2.96 ± 0.14 a	74.1 ± 5.7 a	2.18 ± 0.14 a
Low N concentration large urine patch	73.0 ± 2.9 b	4.05 ± 0.13 bc	114.6 ± 14.9 a	3.22 ± 0.06 b
High N concentration large urine patch	74.4 ± 2.1 b	4.32 ± 0.10 cd	127.7 ± 17.3 a	3.97 ± 0.19 c
Four low N concentration small urine patches	71.4 ± 1.6 b	3.82 ± 0.10 b	106.4 ± 1.1 a	3.05 ± 0.05 b
Four high N concentration small urine patches	92.0 ± 4.0 c	4.71 ± 0.03 d	107.3 ± 16.6 a	4.26 ± 0.16 c
ANOVA *P* value	< 0.001	< 0.001	0.12	< 0.001

Increasing the N loading rate in four smaller urine patches increased pasture biomass ($p < 0.01$) 2 weeks after urine application and increased foliar N content ($p < 0.01$) on both sampling occasions. Patch size had no effect ($p > 0.05$) on pasture biomass or foliar N content when applied at 200 kg N ha^{-1}, either 2 or 4 weeks following urine deposition. At 800 kg N ha^{-1}, the biomass was greater ($p < 0.05$) in four small urine patches compared to one large urine patch after 2 weeks, however, no differences ($p > 0.05$) were observed after 4 weeks. No differences ($p > 0.05$) were observed between the foliar N content of four small patches compared to one large urine patch, when urine was applied at 800 kg N ha^{-1}.

4.3.3 N_2O fluxes from artificial urine-influenced soil

The results for the N_2O emissions over the course of the experiment can be seen in Fig. 4.3b, alongside the weather station data in Fig. 4.3a and the soil water-filled pore space (WFPS; calculated from the SDI-12 soil moisture sensors) in Fig. 4.3c. The initial peak in N_2O emissions were similar across all treatments where urine was applied, indicating initially the N content was not limiting N_2O production. Following the maxima of this initial peak, the two high N treatments begin to branch off from the low N treatments, and generally remained higher than the low N containing treatments for ca. 2 weeks. The major peaks in N_2O emissions occurred just after rainfall events (Fig. 4.3a and b). The largest N_2O peaks were observed for the high N containing small urine patches, which peaked at around 150 μg N_2O-N m^{-2} h^{-1}.

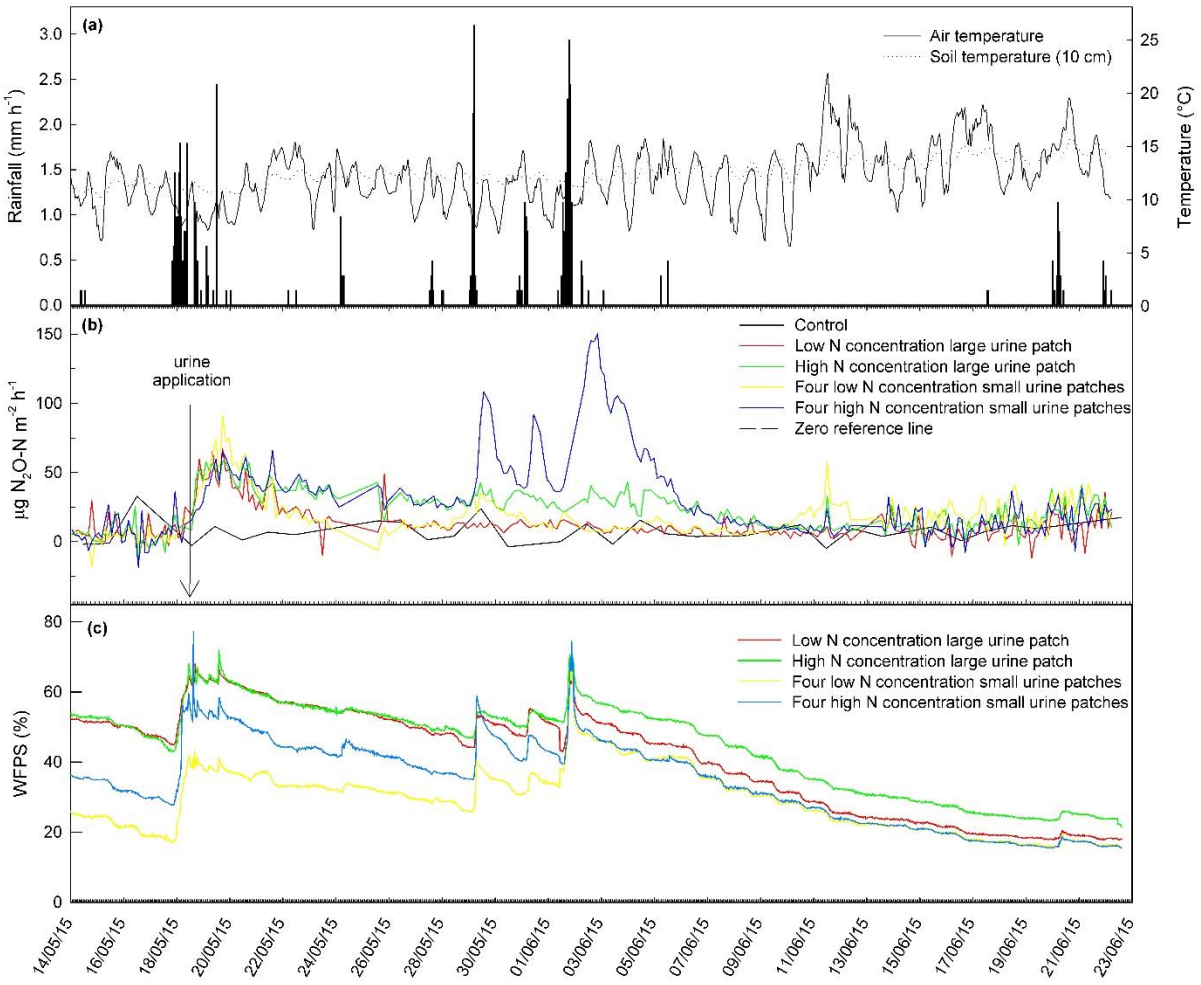

Figure 4.3 Weather station data (panel a), nitrous oxide emissions (panel b) and water-filled pore space (WFPS; panel c) following application of artificial sheep urine of varying N concentration and patch size to a Eutric Cambisol. Figure legends apply to each respective panel, for both panels b and c, where the traces represent the treatment mean (n = 3).

4.3.4 Cumulative N_2O emissions

Cumulative N_2O emissions (mg N_2O-N 0.05 m^{-2}) over the 39 day measuring period (Fig. 4.4) followed the numerical trend high N small urine patches (6.75 ± 0.54) > high N large patch (4.01 ± 0.57) > low N small patches (2.56 ± 2.40) > low N large patch (1.36 ± 0.44) > control (0.36 ± 0.07). The variability in cumulative N_2O emissions was greater in the low N small urine patches treatment, which arose from one 'high emitting' replicate.

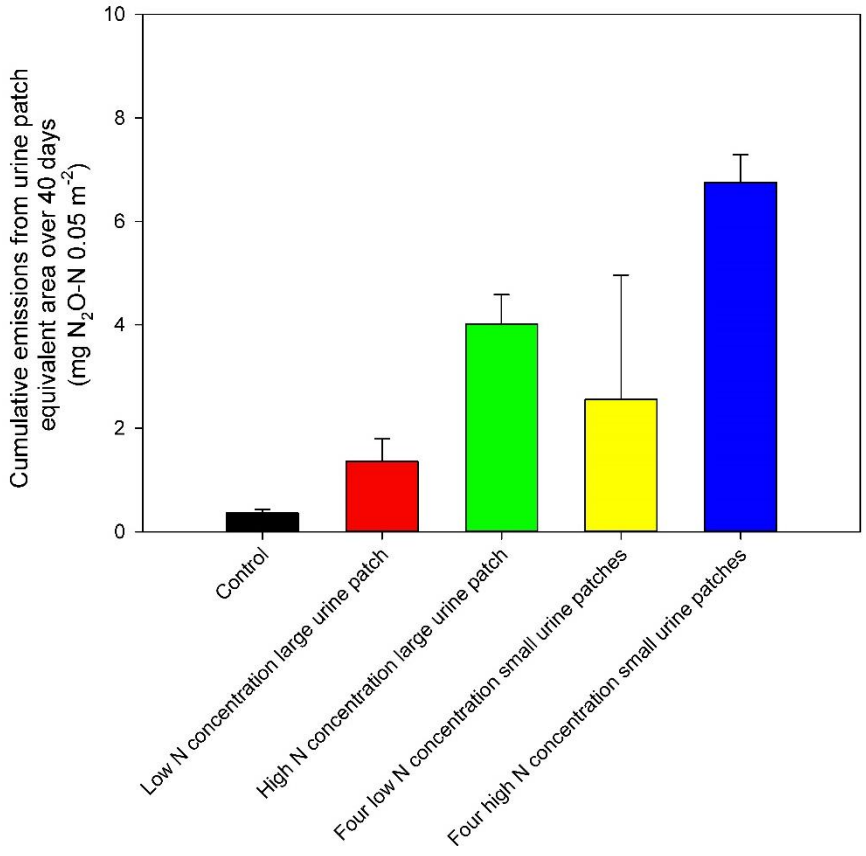

Figure 4.4 Cumulative N_2O-N emissions over the duration of the field trial (40 days), where artificial urine of varying N content and patch size was applied to a Eutric Cambisol. Bars represent means ± SEM (n = 3).

Increasing the N loading rate from 200 to 800 kg N ha^{-1} within a large urine patch increased ($p < 0.05$) cumulative N_2O emissions by a factor of 3, from 1.36 ± 0.44 to 4.01 ± 0.57 mg N_2O-N 0.05 m^{-2} over 39 days. No difference ($p > 0.05$) was observed between cumulative N_2O emissions when increasing N concentration in the four small sized urine patches. The non-significant effect of increasing N concentration on cumulative N_2O emissions in the small urine patches is likely to be the result of the high N_2O emitting replicate; if this replicate is removed from the analysis the difference then becomes significant ($p < 0.01$), although the potential for such high emissions cannot be ruled out from smaller urine patches.

Increasing the number of replicates per treatment is recommended in order to capture spatial variability of N_2O emissions under field conditions.

The second hypothesis was to determine whether cumulative N_2O emissions were higher from four small compared to one large urine patch (with the same N loading rate). No effect of patch size ($p > 0.05$) was observed between the cumulative emissions at the lower N loading rate (200 kg N ha^{-1}), regardless of whether the high-emitting replicate was included or removed. However, under a higher N loading rate (800 kg N ha^{-1}), cumulative N_2O emissions were greater ($p < 0.05$) from the four small patches (6.75 ± 0.54 mg N_2O-N 0.05 m^{-2} over 40 days) compared to a single large urine patch (4.01 ± 0.57 mg N_2O-N 0.05 m^{-2} over 40 days).

The third hypothesis was that cumulative emissions would be higher from a large urine patch with a low N concentration, as opposed to a small urine patch with a high N concentration (manipulating N loading rate, but keeping total N applied constant). The cumulative emissions from a high N concentration single small urine patch was 1.69 ± 0.14 mg N_2O-N 0.05 m^{-2}, over the duration of the study, where no differences were found ($p > 0.05$) compared to the low N concentration large urine patch, at 1.36 ± 0.44 mg N_2O-N 0.05 m^{-2}.

4.3.5 Emission factors

The N_2O emission factor for the low N concentration large urine patch over 39 days was 0.10 ± 0.05% of the N applied and 0.09 ± 0.02% from the high N concentration large urine patch. The emission factors from the four low N concentration small urine patches was 0.22 ± 0.24% of the applied N, where one replicate had the highest emission factor of 0.69%, highlighting the large variability observed in this treatment. The N_2O emission factor from the four high N concentration small urine patches was 0.16 ± 0.01% of the total N applied and the emission factor from the high N concentration large urine patch was 0.09 ± 0.02% of the N applied. No effect of patch size on N_2O emission factors was observed in the low N

concentration treatments, however, the emission factor was significantly greater ($p < 0.05$) from the four small urine patches compared to a single large urine patch, at 800 kg N ha^{-1}. No significant differences in N_2O emission factors were observed when increasing N concentration from 4–16 g N l^{-1} either from four small patches, or from a single large patch.

4.3.6 Effect of reduced sampling frequency on cumulative N_2O emissions

When sampling frequency was reduced to one flux measurement per day (compared to 8 flux measurements per day), between the hours of 10 a.m. and 2 p.m., the mean difference in cumulative emissions was within 1 ± 6% of the high frequency measurement value, although differences between individual replicates ranged from an underestimate of 39% and an overestimate of 50% of the value obtained by taking 8 flux measurements per day. Sampling N_2O fluxes once a week resulted in a mean underestimation of the cumulative emissions of 54 ± 24%, and individual replicates ranged from an underestimate of 268% and an overestimate of 37%. The last sampling regime comparison was to measure N_2O fluxes three times in the first week following urine application, twice in the second week, and once per week thereafter. Across all treatments and replicates, this sampling regime would have underestimated emissions by 22 ± 10% of the high frequency value, and individual replicates ranged from an underestimate of 90% to an overestimate of 38%.

4.4 Discussion

When urine was applied at the same N loading rate, NO_3^- concentrations did not peak as high, and returned to control values faster in four smaller urine patches compared to a single large patch. The observed effect could have been due to the larger wetted perimeter created by the four small urine patches (179 cm) in comparison to one large urine patch (89 cm). This may have allowed increased plant access to the nutrient rich patch via roots and stolons, which may

explain the greater plant biomass and foliar N content when increasing urine N loading rate in smaller urine patches. Diffusion away from the urine patch edge has been identified as an important mechanism for N processing and transformations in urine patches of different sizes (Orwin et al., 2009). The difference in patch sizes may have allowed a greater lateral, as opposed to vertical, diffusion of soluble N and C into surrounding soil, potentially influencing differing proportions of nitrifying and denitrifying microorganisms. A greater diffusion of NO_3^- beyond the area of immediate urine application may have also resulted in the lower peak of NO_3^- found in smaller urine patches, as soil cores were only taken where urine had been directly applied, and Rhizon samples were taken at the centre of the urine patch. The soil mineral N concentration remained higher for a longer period with increasing urine N concentration, which means N losses can occur under partially differing environmental conditions from the high N treatment compared to the lower N treatment (Dijkstra et al., 2013).

Concentrations of NO_3^-, NH_4^+, total N and DOC in the urine patches (measured in both the soil extracts and the soil solution) tended to vary widely, having large standard errors. This large spatial variability in factors which can drive N_2O emissions, is likely to have contributed to the large observed variation in N_2O emissions in this study. This could also be problematic when comparing urine patch soil properties from duplicated plots with N_2O emissions from chambers, as processes may be occurring at different rates in the different locations. We suggest the development of non-destructive sampling techniques for monitoring soil conditions within chambers to ensure monitored soil properties accurately reflect conditions within chambers.

In this study, increasing urine N loading rate from 200 kg N ha^{-1} to 800 kg N ha^{-1} generally increased cumulative N_2O emissions, but the emission factors were not significantly different. This is similar to the finding of Selbie et al. (2014), who found a curvilinear increase in cumulative N_2O emissions upon increasing N loading rate. Increasing urine N loading rate,

however, had no effect on the fraction of N_2O produced under laboratory (van Groenigen et al., 2005a) and field conditions (van Groenigen, 2005b; Dai et al., 2013; Luo et al., 2013). However, urine derived N_2O mission factors have also been shown to decrease (Selbie et al., 2014) or increase alongside N loading rate (Singh et al., 2009). There are contradictory results for the response of urine patch N_2O emission factors to increasing N loading rates and results to date are contradictory and uncertain (Luo et al., 2013; Selbie et al., 2014).

When manipulating urine patch size and N loading rate, but maintaining the same total amount of N applied, no differences were found in cumulative N_2O emissions or emission factors, which is similar to the findings of van Groenigen et al. (2005a). Cumulative N_2O emissions and emission factors, however, were significantly greater from four small urine patches compared to one large urine patch, when applied at 800 kg N ha^{-1}. It is difficult to determine a management strategy which would reduce N_2O emissions based on the results of this treatment, as it assumes a difference in the frequency of urine events, but with the same N concentration and total volume of urine excreted. In reality, ruminant urine N concentration and volume varies widely between and within days, and between individuals of the same species (Betteridge et al., 1986; Hoogendoorn et al., 2010; Betteridge et al., 2013). Nevertheless, based on the results of this study and the sheep urine patch sizes used here, it would seem that sheep which urinate more frequently in smaller volumes would emit more N_2O than a sheep urinating less frequently in larger volumes, given the same high urinary N concentration. Further work is required to determine how this effects other N loss processes such as NO_3^- leaching and NH_3 volatilisation, as given the range of patch sizes excreted by cattle, increasing patch size has been shown to logarithmically increase NO_3^- leaching (Li et al., 2012).

It has been recognised that more information is required for values of ruminant urine volume, urine N concentration and urination frequency, where the development of sensor based

technology which can measure all three of these values (e.g. Betteridge et al., 2013), will be important for improving models to predict N losses from grazed grassland. An increase in our knowledge of how ruminant urine N concentration is affected by dietary factors will also be important, e.g. Pacheco et al. (2010) found urinary N concentration of cattle urine to be related to the moisture content, dietary cation-anion difference and soluble sugar content of the forage. Increasing knowledge in these areas will also assist in a better understanding on the effect of differing management and mitigation strategies to reduce N losses (e.g. feed type, grazing intensity, and inclusion of diuretics in the diet).

Increasing urine volume e.g. by the inclusion of NaCl in the diet, has been suggested as a way to reduce N losses as it would reduce N loading rate per urination, potentially promoting a better dispersion of N across the pasture (Costall and Betteridge, 2010; Pacheco et al., 2010), although this will depend on livestock movement and behaviour. The results of our study, however, have shown that N_2O emission were no different in patches of differing N loading rates, for the same given amount of N applied. Therefore, for a reduction in N_2O emissions from sheep urine patches, it is suggested that a reduction in dietary N would need to occur concurrently with any mitigation strategies based on increasing total urine volume.

Both Ledgard et al. (2007) and Liu and Zhou (2014) show that increasing NaCl in the diet of cattle and sheep, respectively, results in increased urine volume and frequency, but the average volume of individual urine events remained similar. Both Li et al. (2012) and Betteridge et al. (2013) have shown that considering mean values (rather than varied values) for cattle urine volume, frequency and N concentration causes differences of ca. 5-10% in modelled NO_3^- leaching losses. The use of mean values for urine volume and N concentration are also often used in plot based studies of urine patch N_2O emissions, which may also cause inaccuracies when upscaling such emissions.

Across all urine treatments, N_2O emission factors were on average ten times lower than that of the 1% default IPCC emission factor for sheep excreta (IPCC, 2006). In a large study based on numerous field trials conducted across New Zealand, the mean sheep urine patch emission factors from lowland pasture soils was also found to be lower than the default IPCC emission factor for excreted N at pasture, at 0.55% of the applied N (Kelliher et al., 2014). The low emission factors found in this study may have been caused by the relatively dry soil conditions which prevailed during the study, or due to the short period of measurement (i.e. a year is the normal requirement for calculation of emission factors). Low emission factors have been previously observed above sheep grazed pastures under dry summer months, with maximum emissions occurring under cooler and wetter soil conditions (Allen et al., 1996; Saggar et al., 2007). Rainfall and soil drainage class have been identified as key variables influencing N_2O emission factors from urine returns (de Klein et al., 2003), and a consideration of environmental and management factors (which are not considered in the IPCC Tier 1 approach) will undoubtedly be important for better constraining N_2O emission estimates from grazed soils.

The values for emission factors in this study should be considered with care, as they were derived from artificial urine and overall emissions were fairly low and variable, which may have masked some effects. It is suggested that similar studies should be conducted under differing environmental conditions to determine if the effects hold true. Real urine is generally advocated for the calculation of emission factors (deKlein et al., 2003), however, the use of artificial urine was beneficial in this study as it allowed the manipulation of N concentration. The artificial urine contained the recommended constituents for adequate representations of urine for N_2O emissions (Kool et al., 2006) and the proportion of urea was increased to manipulate the N content, which is consistent with an increase in the proportion of urea

excreted by ruminants occurring as a result of increased dietary crude protein (Dijkstra et al., 2013).

Our results show that daily sampling of gases between 10 a.m. and 2 p.m. resulted in a good approximation of mean N_2O emissions. Sampling more often directly after urine application and tapering the sampling towards the end of the experiment resulted in a better approximation of emissions than weekly sampling, although both sampling strategies had a high variability and resulted in a mean underestimation of emissions. Additional sources of error which may occur during manual gas sampling, which were not assessed here, include errors associated with vial evacuation, filling and storage prior to analysis of the gases. Using an automated system, with samples being immediately pumped to a GC, eliminates the potential for such errors.

4.5 Conclusions

Cumulative N_2O emissions generally increased, but emission factors remained similar, with increasing N loading rate (200–800 kg N ha^{-1}). Emission factors were similar from a low N concentration large urine patch (200 kg N ha^{-1}) compared to a high N concentration small urine patch (800 kg N ha^{-1}), when applying the same total amount of N. However, emissions were larger from four smaller patches compared to one large urine patch under the same N loading rate (800 kg N ha^{-1}). These conclusions suggest a reduction in overall dietary N would need to occur alongside any mitigation strategies which manipulate urine volume, in order to be effective at reducing N_2O emissions from sheep urine patches. For the same total volume and concentration of N excreted in the urine, sheep which urinate little and often may be causing greater emissions than sheep which urinate less frequently with a larger volume and patch size. With this in mind, further research is required to gather more data on typical sheep urine volume (both individual urine events and daily volumes), frequency and N

concentrations. Such parameters may vary due to sheep breed, edaphic and climatic conditions, diet and management or mitigation practices (e.g. inclusion of salt in diet). Improving our knowledge on the links between the plant-animal-soil–atmosphere systems will be important for determining more accurate emission estimates from sheep grazed grasslands, and for determining the efficacy of N_2O mitigation strategies.

4.6 Acknowledgements

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Chapter 5

The mobility of nitrification inhibitors under simulated ruminant urine deposition and rainfall: a comparison between DCD and DMPP

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Abstract

Urine patches within pasture soils are hotspots for N cycling and losses, where nitrification inhibitors (NI) offer a means of reducing such losses. Within urine influenced soil, more research has been conducted on the effectiveness of dicyandiamide (DCD) than 3,4-dimethylpyrazole phosphate (DMPP) as NI. Differences in the efficacy of these NI are often ascribed to a greater mobility of DCD, which may lead to spatial separation from NH_4^+ and nitrifying microorganisms. We tested the mobility of ^{14}C-labelled DCD and DMPP relative to sheep urine derived NH_4^+ in soil columns of contrasting texture and organic matter content, following simulated rainfall. We also assessed factors influencing the vertical mobility of these NI in soils, including solubility, sorption/desorption processes and microbial degradation and uptake. Following 40 mm rainfall, without the presence of sheep urine, the distribution of both NI were similar in the soil columns, however, there was a greater retention of DCD compared to DMPP in the top 1 cm. Both NI appeared to co-locate well with urine-derived NH_4^+, and the presence of sheep urine altered the leaching profile of the NI (compared to rainfall application alone), but this effect was inhibitor and soil-type dependent. A greater sorption to the soil matrix was observed for DCD in comparison to DMPP in all three studied soils, and the presence of urine generally increased desorption processes. Of the NI applied to the soil columns, 18-66% was taken up within 30 min by the microbial community. However, only small amounts (<1%) were mineralized during this period. In conclusion, due to the greater adsorption of DCD as opposed to DMPP and similarity in the degree of co-location of both NI with urine NH_4^+, the results of this study suggest that differences in microbial uptake and degradation may be more important parameters for explaining differences in the efficacy of reducing nitrification. Further work is required to determine the comparative efficacy of both NI in reducing nitrification rates under field conditions in a range of soil types and environmental conditions.

Key Words: grazed grassland; livestock ecosystems; nitrogen use efficiency; nutrient dynamics

5.1 Introduction

In pasture soils, high loadings of nitrogen (N) are deposited within ruminant urine patches and these sites are particularly vulnerable to losses of N to the environment. Typically, 20% of deposited urinary-N is leached as NO_3^-, 13% is volatilised as NH_3 and 2% is emitted as the greenhouse gas N_2O (Selbie et al. 2015). While N_2O constitutes a small agronomic loss in terms of magnitude of N, having nearly 300 times the global warming potential of CO_2 (IPCC 2007), it accounts for 46% of agricultural greenhouse gas emissions (Smith et al. 2007). The agricultural sector will need to contribute to decreasing emissions (Misselbrook et al. 2014) in order to achieve targets (80% reduction from 1990 baseline levels by 2050) set by the UK, and other governments. Reducing N loss via NO_3^- leaching and improving N use efficiency would translate to a direct economic benefit for farmers and reducing N_2O emissions from grasslands could contribute to decreasing emissions from the livestock sector.

Nitrification inhibitors (NI) are a potential mitigation strategy which can reduce losses of N from urine patches deposited to grassland soils (Di and Cameron 2012; Ledgard et al. 2014; Luo et al. 2015). By delaying the conversion of NH_4^+ to NO_3^-, the opportunity for plant acquisition, immobilization, fixation and adsorption of NH_4^+ is increased (Di and Cameron 2007). This can potentially reduce emissions of N_2O from both nitrification and denitrification (Gilsanz et al., 2016), where nitrification has been shown to be the dominant N_2O producing process in soils with a WFPS of 35-60%, but at a higher WFPS (70%), denitrification becomes the dominant N_2O producing process (Bateman and Baggs 2005). By the same processes, NI can also reduce the amount of NO_3^- available for leaching (Di and Cameron 2004).

Two of the most widely used NI are dicyandiamide (DCD) and dimethylpyrazole-phosphate (DMPP) (Liu et al. 2013). DCD blocks the electron transport chain in the cytochrome of ammonia monoxygenase (AMO), whereas DMPP binds indiscriminately to the membrane-bound AMO (Chaves et al. 2006; Fiencke and Bocke 2006; Benckiser et al. 2013), delaying the first and rate-limiting step of nitrification (the oxidation of NH_4^+ to NO_2^-; Zerulla et al. 2001). In comparison to DCD, DMPP has been shown to be less phytotoxic, and lower application rates are required (Wissemeier et al. 2001; Zerulla et al. 2001; Di and Cameron 2012). Both NI have demonstrable efficacy in reducing N losses from fertilizer applications (Weiske et al. 2001; Liu et al. 2013) and livestock slurry (Fangueiro et al. 2009; Pereira et al. 2010), however, DCD applications to urine patches have been more widely researched (e.g. Di and Cameron 2003; O'Callaghan et al. 2010; de Klein et al. 2011) in comparison to DMPP (e.g. Di and Cameron 2011; Di and Cameron 2012). Some authors have found a difference between the efficacy of DCD and DMPP e.g. Weiske et al. (2001) found DMPP to be reduce N_2O emissions from fertilizer by an average of 49%, whereas DCD reduced emissions by an average of 26%. Di and Cameron (2012), however, found that DCD and DMPP reduced N_2O emissions by a similar amount from cattle urine (62 and 66% reduction, respectively). The efficacy of NI in reducing N_2O emissions and NO_3^- leaching can vary widely. In a laboratory study of nine contrasting UK soils, the efficacy of DCD to inhibit NH_4^+ oxidation, net NO_3^- production and emissions of N_2O was lower in soils of high temperature, clay content and organic matter content (McGeough et al. 2016). Differences in efficacy have been attributed to a lower mobility of DMPP in comparison to DCD, due to a greater sorption of DMPP (Wissemeier et al. 2001; Zerulla et al. 2001; Di and Cameron 2012). Having a high mobility may lead to the spatial separation of NI from NH_4^+ and nitrifying microorganisms (Ruser and Schulz 2015).

Physicochemical characteristics which can influence mobility within soil include solubility and sorption/desorption processes (Carrillo-González et al. 2006). The greater the solubility of a chemical in water, the greater the potential for vertical transport. The sorption/desorption of chemicals within the soil is mainly influenced by the organic matter content of soil, where charges associated with the chemical influences the types of bond established. DCD is a net neutrally charged molecule and binding takes place on the surface of organic matter, through hydrogen bonding of the $–NH_2$ and =NH functional groups to negative carboxyl groups of organic matter (Zhang et al. 2004; Singh et al. 2008; Shepherd et al. 2012). Conversely, DMPP is positively charged and adsorption is thought to occur to the mineral fraction of soils, such as clays and silts (Barth et al. 2001, 2008). As chemicals are transported through soil, further biological transformations may influence the quantity of substance available for vertical movement e.g. microbial uptake and mineralization. As DMPP is a heterocyclic compound, it is not readily degradable, resulting in a slower degradation rates in soil in comparison to DCD (Weiske et al. 2001; Chaves et al. 2006). In the specific conditions of urine influenced soils, the high concentration of NH_4^+, K^+ and other cations, may saturate cation exchange sites leading to further movement of NI down the soil profile.

The objective of this study was to obtain information on how the combination of NI characteristics and soil conditions can affect the mobility and co-location of NH_4^+ and NI in soils, with ruminant urine as the source of NH_4^+. We investigated physicochemical (solubility and sorption/desorption) and biological (microbial uptake and degradation) factors influencing the vertical mobility of DCD and DMPP, in soil columns of contrasting texture and organic matter following a 40 mm rainfall event, with and without the presence of sheep urine. We hypothesised that 1) DCD would move further down the soil profile than DMPP following simulated rainfall, due to a greater sorption of DMPP, 2) a greater co-location would be observed for DMPP with urine NH_4^+, due to the lower mobility of DMPP, 3) the presence of

sheep urine would increase vertical movement and desorption of both NI, due to saturation of soil exchange sites by ions within sheep urine and 4) a greater microbial uptake and mineralization would occur for DCD in comparison to DMPP, due to a greater bioavailability of DCD in comparison to DMPP.

5.2 Materials and methods

5.2.1 Soil and sheep urine analysis

Three soil types were selected for experimentation, on the basis of contrasting texture and organic matter content: a sandy loam textured Eutric Cambisol (53°24′N, 4°02′W), a sandy clay loam textured Eutric Cambisol (53°14′N, 4°01′W) and a high organic matter containing Sapric Histosol (52°52′N, 0°47′W). Both Eutric Cambisol samples were collected from beneath moderately sheep grazed and fertilised pasture, and the Sapric Histosol was collected from a eutrophic lowland peat used in intensive arable agriculture. The soil types used in this study had not been previously exposed to either DCD or DMPP. A summary of soil properties is presented in Table 1.

Soil was sampled in triplicate (0–10 cm depth), sieved (< 2 mm) in order to reduce sample heterogeneity and stored at 4°C until required. Soil moisture content was determined by weight difference after oven drying (105°C), and organic matter was determined on dry soil by loss-on-ignition in a muffle furnace (450°C; Ball 1964). Soil C:N ratio was determined on oven-dried, ground soil samples using a TruSpec® Analyzer (Leco Corp., St. Joseph, MI). The cation exchange capacity (CEC) of soils was determined using an unbuffered salt extraction method of Schofield (1949). Soil pH and electrical conductivity (EC) were measured using standard electrodes (1: 2.5 (w/v) soil-to-distilled water). Soluble C and N were determined in 1:5 soil-to-0.5 M K_2SO_4 extracts using a Multi N/C 2100S Analyzer (AnalytikJena, Jena, Germany), within 24 h of sample collection, according to Jones and Willett (2006). Microbial

C and N were determined using the chloroform fumigation-extraction method of Voroney et al. (2008) (K_{EC} = 0.35 and K_{EN} = 0.5). Total available nitrate (NO_3^-), ammonium (NH_4^+) and phosphate (P) were determined within 0.5 M K_2SO_4 extracts via the colorimetric procedures of Miranda et al. (2001), Mulvaney (1996) and Murphy and Riley (1962), respectively. Cations (Na^+, K^+ and Ca^{2+}) were determined within 1:5 (w/v) soil-to-1 M NH_4Cl extracts using a Sherwood Model 410 Flame Photometer (Sherwood Scientific Ltd, Cambridge, UK).

Sheep urine was collected from Welsh Mountain ewes fed a diet of 80% *Lolium perenne* L. and 20% *Trifolium repens* L., where urine was collected between the hours of 10 a.m. and 4 p.m. Several urine samples from a single sheep were pooled for experimental use. The urine was frozen (unacidified) before use to avoid losses of N. The sheep urine had a pH of 8.99 and an EC of 22 mS cm^{-1}; the urine contained a total of 2.27 g N l^{-1}, 3.00 g organic C l^{-1}, 1.71 g urea N l^{-1}, 44.9 mg NH_4^+-N l^{-1}, 0.44 mg NO_3^--N l^{-1}, 0.92 mg P l^{-1}, 7.16 g K l^{-1}, 1.11 g Na l^{-1} and 73.3 mg Ca l^{-1}. Properties were measured directly on the urine via the methods described above and urea was measured using the method of Orsonneau et al. (1992).

Table 5.1 Properties of soils used to fill the soil columns, values represent means ± SEM, *n* = 3, letters indicate significant differences between the soils, results are reported on a dry soil weight basis and N.D. stands for not determined.

Soil Property	Sandy Loam	Sandy Clay Loam	Sapric Histosol
Texture: Sand (%)	80.0 ± 2.52 a	49.0 ± 2.00 b	N.D.
Silt (%)	11.7 ± 2.19 a	31.3 ± 0.88 b	N.D.
Clay (%)	8.33 ± 0.67 a	19.7 ± 1.20 b	N.D.
Organic matter (%)	5.40 ± 0.29 a	5.10 ± 0.44 a	77.6 ± 0.36 b
Gravimetric moisture content (%)	14.2 ± 0.12 a	19.5 ± 0.33 b	61.2 ± 0.10 c
Cation exchange capacity (meq 100 g^{-1})	14.0 ± 0.29 a	14.8 ± 0.68 a	80.8 ± 0.80 b
pH	6.64 ± 0.15 a	6.42 ± 0.04 a	6.37 ± 0.06 a
Electrical conductivity (µS cm^{-1})	51.1 ± 13.5 a	67.8 ± 7.34 ab	107 ± 11.8 b
Total C (g kg^{-1})	24.1 ± 2.50 a	19.1 ± 0.76 a	392 ± 1.33 b
Total N (g kg^{-1})	3.29 ± 0.14 a	3.12 ± 0.13 a	26.6 ± 0.09 b
Dissolved organic C (mg C kg^{-1})	52.7 ± 2.57 a	64.7 ± 13.4 a	959 ± 79.1 b
Total dissolved N (mg N kg^{-1})	7.51 ± 0.78 a	9.92 ± 0.66 a	71.4 ± 6.98 b
Microbial C (g kg^{-1})	0.35 ± 0.01 a	0.38 ± 0.04 a	4.41 ± 0.20 b
Microbial N (mg kg^{-1})	27.2 ± 0.79 a	26.1 ± 1.33 a	777 ± 42.3 b
Extractable NH_4^+ (mg N kg^{-1})	0.20 ± 0.01 a	0.24 ± 0.03 a	6.50 ± 0.46 b
Extractable NO_3^- (mg N kg^{-1})	0.61 ± 0.14 a	0.89 ± 0.12 a	29.3 ± 2.73 b
Extractable P (mg kg^{-1})	0.32 ± 0.004 a	0.08 ± 0.04 a	26.1 ± 3.59 b
Exchangeable K (meq kg^{-1})	2.39 ± 0.43 a	1.61 ± 0.31 a	8.95 ± 3.79 a
Exchangeable Na (meq kg^{-1})	0.77 ± 0.08 a	1.37 ± 0.08 a	5.17 ± 0.51 b
Exchangeable Ca (meq kg^{-1})	51.8 ± 1.51 a	53.3 ± 2.12 a	617 ± 17.4 b

5.2.2 Comparative mobility of ^{14}C-DCD and ^{14}C-DMPP under simulated rainfall

To compare the mobility of [U]^{14}C-DCD and 5-^{14}C-DMPP (American Radiolabelled Chemicals, St Louis, MO, USA) in contrasting soils under a simulated rainfall event, polypropylene tubes (*n* = 3; 15 cm depth; 0.8 cm diameter) were repacked with sieved, field moist soil (soil sampled at 0-10 cm depth) to approximate field bulk density values (9, 8 and 5 g for the sandy loam, sandy clay loam and Sapric Histosol). This resulted in bulk densities of 1.0, 0.9 and 0.4 g cm^{-3} and porosities of 0.60, 0.67 and 0.71 in the sandy loam, sandy clay loam and Sapric Histosol soil columns, respectively (particle density was assumed to be 2.65 g cm^{-3} in the mineral soils and 1.4 g cm^{-3} in the organic soils; Rowell 1994). In order to pack the soils evenly, additions of soil to the columns were made at increments, followed by lightly tapping the columns at the base to create an even density. The bottom of the tubes contained nylon mesh, to allow for drainage of leachate and to prevent any loss of soil. Nevertheless, no leachate was present following the rainfall simulations. Field relevant application rates of either ^{14}C-DCD (1 g l^{-1}; 50 µl; *ca.* 1 kBq) or ^{14}C-DMPP (0.1 g l^{-1}; 50 µl; *ca*. 1 kBq) were applied to the top of the column and a 40 mm rainfall event was simulated by adding 2 ml of distilled water drop-wise to the soil columns, *ca*. 5 minutes after the application of the NI. This rainfall event was chosen to simulate UK storm conditions which promote rapid water movement down the soil profile. It should also be noted that these leaching rates also approximate rates of water movement down preferential flow pathways in the soil profile under lower rainfall events. Preliminary studies indicated that the wetting front generally reached, but did not exceed the soil column length (15 cm).

The soil columns were incubated for 0.5 h, at laboratory temperature, after which the tubes were cut into the following depth fractions with a scalpel: 0-1, 1-2, 2-3, 3-5, 5-7, 7-9, 9-12 and 12-15 cm. The entire cut sections (including tube, to extract soil adhered to inner edge) were extracted with 0.5 M K_2SO_4 (1:5 w/v; 0.5 h; 150 rpm). An aliquot (1.5 ml) of the soil

solution was centrifuged (14 000 *g*; 5 min) and the resulting supernatant was mixed with HiSafe 3 scintillant (PerkinElmer, Llantrisant, UK) and the activity measured using a Wallac 1404 Liquid Scintillation Counter (Wallac EG&G, Milton Keynes, UK).

5.2.3 Effect of ruminant urine on NI mobility and co-location of NI with urine ammonium

To determine if the presence of urine influences the vertical movement of DCD or DMPP, soil columns (n = 3) were prepared and processed as above. A sheep urine deposition event was simulated *ca.* 2 minutes following application of the ^{14}C-DCD or ^{14}C-DMPP and preceding the simulated rainfall event, by applying 250 µl of sheep urine to the top of the soil column. The vertical distribution of the NI was compared to soil columns incubated without urine.

The NH_4^+ concentration of the 0.5 M K_2SO_4 extracts of each depth fraction was also determined on the urine treated soil columns, via the method described previously, to determine the co-location of ^{14}C-DCD and ^{14}C-DMPP with urine-derived NH_4^+.

5.2.4 Solubility of DCD and DMPP in water

To determine the water solubility of DCD and DMPP, the OECD (1995) flask method was used. Briefly, 5 g of NI was added to 10 ml of water (n = 3) and incubated at 30ºC on a rotary shaker for 24 h. One replicate was then removed and incubated at 20ºC for 24 h with occasional shaking, before centrifuging at 10 000 *g*. Samples were syringe filtered (0.2 µm) and analysed for total dissolved C, as described above, and the amount of NI dissolved in the water calculated. One of the remaining replicates was incubated for another 24 h at 30ºC and the final replicate was incubated for an additional 48 h, before incubation at 20ºC for a further 24 h and preparation of samples for analysis of dissolved C. This was conducted to ensure additional time had no effect on the amount of NI dissolved.

5.2.5 Sorption and desorption

Sorption isotherms were determined for ^{14}C-DCD and ^{14}C-DMPP in the three contrasting soils in the presence and absence of sheep urine,, using similar methods as Jones and Brassington (2003). Briefly, ^{14}C-DCD or ^{14}C-DMPP was applied (50 µl; *ca*. 1 kBq) to 0.5 g (n = 3) of field moist soil, following which 2.5 ml of either 0.01 M $CaCl_2$ or sheep urine was added to the soils. A total of 8 concentrations of ^{14}C-DCD and ^{14}C-DMPP were used, ranging from 0.08-10 mg NI l^{-1}. The soil suspensions were shaken for 0.5 h at 150 *rpm* on a rotary shaker, subsequently an aliquot (1.5 ml) was centrifuged (10 000 *g*; 5 min) and the ^{14}C activity in the supernatant determined by liquid scintillation counting, as above. Sorption of NH_4^+ was also assessed as described above, using 8 concentrations ranging from 2.3-300 mg NH_4^+-N l^{-1}, in 0.01 M $CaCl_2$. The NH_4^+ concentration in the supernatant was determined as above. The partition coefficient (K_d) for the NI (with and without the presence of urine) and NH_4^+ within soil, was determined via Equation 1, where C_{ads} (µmol kg^{-1}) is the concentration adsorbed to the soil solid phase at equilibrium and C_{sol} (µmol l^{-1}) is the adsorbate concentration remaining in solution at equilibrium.

$$K_d = C_{ads} / C_{sol} \qquad \text{(Eqn. 5.1)}$$

Desorption, with and without the presence of sheep urine, was determined by adding either ^{14}C-DCD (25 µl; *ca*. 0.5 kBq) or ^{14}C-DMPP (25 µl; *ca*. 0.5 kBq) at two concentrations (1 and 10 mg l^{-1}) to 0.2 g of soil (n = 3). Four successive washes of the soil were conducted by adding either 1 ml of 0.01 M $CaCl_2$ or sheep urine and by conducting a final wash with 0.5 M K_2SO_4. Samples were shaken for 0.5 h at 150 *rpm* on a rotary shaker between the additions of fresh wash solution. At the end of each wash period the samples were centrifuged (10 000 *g*; 5 min) and the supernatant removed prior to adding fresh 0.01 M $CaCl_2$ or sheep urine. The

activity in the supernatant was determined as described previously, where the activity residing in the entrained solution trapped within the soil matrix was accounted for. After the final wash, soils were dried (105 °C; 24 h) and ground before combustion in an OX400 biological oxidizer (RJ Harvey, Hillsdale, NJ, USA), where evolved $^{14}CO_2$ was captured in Oxysolve C-400 (Zinsser analytic, Frankfurt, Germany) to quantify ^{14}C remaining bound to soils following washes.

5.2.6 Substrate mineralization and microbial uptake

Mineralization and microbial uptake of ^{14}C-DCD and ^{14}C-DMPP in the three soils was determined to quantify the degradation of the NI during the course of the incubation, via the methods of Hill et al (2007). 0.3 ml of ^{14}C-DCD or ^{14}C-DMPP (*ca.* 0.5 kBq ml^{-1}; 0.1 and 1 g l^{-1}) were added to 1 cm^3 of soil (n = 3), contained in 10 cm^3 glass vessels. Evolved $^{14}CO_2$ was captured by flowing (*ca*. 100 ml min^{-1}) moist air over the soil surface, with the outflow passing through two consecutive 0.1 M NaOH traps (capture efficiency > 95%; Hill et al. 2007). Traps were changed after 0.05, 0.12, 0.25, 0.5, 1, 2, 4 and 8 h, and the activity in the solution determined by liquid scintillation counting as above.

To enable calculation of the ^{14}C-NI pool taken up by soil microbes, ^{14}C-DCD or ^{14}C-DMPP (0.3 ml; *ca.* 0.5 kBq; 0.1 and 1 g l^{-1}) was pipetted evenly onto the soil surface (1 g; n = 3) and extractions using ice-cold 0.5 M K_2SO_4 (1:5 w/v) were conducted at 0.05, 0.12, 0.25, 0.5 and 1 h following addition of the substrate. The soils were shaken (150 rpm; 0.5 h) and subsequently centrifuged (10 000 *g*, 10 min). The ^{14}C in the resulting supernatant was determined via liquid scintillation counting as described above. Uptake of the substrate by soil microbes was calculated by deducting the 0.5 M K_2SO_4 extractable pool from the starting ^{14}C pool. This is assuming the extraction procedure removed all exchangeable ^{14}C-NI and the remainder was taken up into the microbial biomass.

5.2.7 Statistical analysis

To compare the vertical mobility of ^{14}C-DCD and ^{14}C-DMPP under rainfall, to determine how urine influences the vertical mobility of the NI and to compare the co-location of the NI with that of urine-NH_4^+, a one-way ANOVA with Tukey's post-hoc test was used to compare each section depth, following testing for normality (Ryan-Joiner test) and homogeneity of variance (Levene's test). The same analysis was conducted on the slope of the linear sorption isotherms, following log transformation of data. A one-way ANOVA was also conducted for desorption after the fourth wash in either urine or $CaCl_2$, for mineralization at the 8 h time point and microbial uptake after 1 h. All statistical analyses were performed in Minitab 17.1.0 (Minitab Inc., State College, PA).

5.3 Results

5.3.1 Comparative vertical mobility of NI following simulated rainfall

The distribution of extractable DCD-^{14}C and DMPP-^{14}C following simulated rainfall was generally similar within each soil type (Fig. 1). However, a greater retention of ^{14}C-DCD was observed in comparison to ^{14}C-DMPP in the top 0-1 cm depth fraction of the sandy loam (Fig. 1a) and sandy clay loam columns (Fig. 1b). The total amount of DCD-^{14}C extracted from the columns with 0.5 M K_2SO_4 after 0.5 h was 66.6 ± 1.29, 63.9 ± 0.65 and 38.8 ± 0.53% of that applied to the sandy loam, sandy clay loam and the Sapric Histosol, respectively. In comparison, the percentage of DMPP-^{14}C extracted from the columns was 79.4 ± 2.14, 72.5 ± 1.42 and 39.1 ± 1.39% of that applied to the sandy loam, sandy clay loam and Sapric Histosol, respectively.

The presence of sheep urine reduced ($p < 0.01$) the quantity of extractable DCD-^{14}C and DMPP-^{14}C in the top 1 cm of the sandy loam columns (Fig. 1a and d), increased ($p < 0.01$) the amount of extractable DCD-^{14}C in the bottom 12-15 cm depth fraction and had no effect (p

> 0.05) on the extractable amount of DCD-^{14}C and DMPP-^{14}C in each remaining depth fraction. The presence of sheep urine did not influence the extractable amount of DCD-^{14}C or DMPP-^{14}C in any studied depth fraction of the sandy clay loam columns (Fig. 1b and e). The presence of urine had no effect ($p > 0.05$) on the amount of extractable DCD-^{14}C in each depth fraction of the Sapric Histosol (Fig. 1c and f). However, it decreased ($p < 0.001$) the extractable amount of DMPP-^{14}C from the top 1 cm and increased ($p < 0.001$) the extractable amount in the 12-15 cm depth fraction.

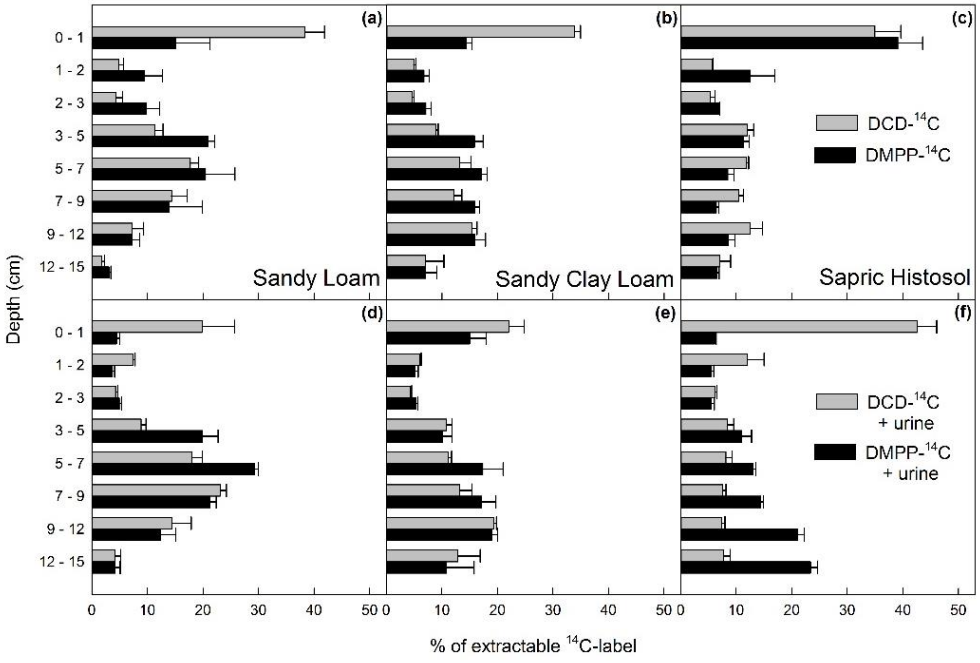

Figure 5.1 The percentage of extractable DCD-^{14}C or DMPP-^{14}C at depth fractions of sandy lam (a and d), sandy clay loam (b and e) and Sapric Histosol (c and f) columns, following a simulated 40 mm rainfall event (a, b and c) and a sheep urine deposition plus a 40 mm rainfall event (d, e and f). Soil type labels apply to each column and legends apply to each row of panels. Bars represent means (n = 3) and error bars represent SEM.

The percentage of applied DCD-^{14}C extracted from the soil columns with applied sheep urine plus rainfall was 79.6 ± 6.58, 72.9 ± 1.92 and 43.6 ± 0.73% of the added label applied to the sandy loam, the sandy clay loam and Sapric Histosol, respectively. The total amount of DMPP-^{14}C extracted from the soil columns under the same conditions was 79.4 ± 0.77, 71.5 ± 3.54 and 47.9 ± 0.01% in the sandy loam, sandy clay loam and Sapric Histosol, respectively. In conclusion, urine increased the total amount of DCD extracted from soils, but had no effect on DMPP.

5.3.2 Co-location of NI with urine-derived ammonium

In general, the distribution of both DCD-^{14}C and DMPP-^{14}C within the soil profile coincided well with the urine-derived NH_4^+ (Fig. 2). A greater ($p < 0.001$) percentage of total column extractable DCD-^{14}C in comparison to NH_4^+ was found in the top 1 cm in all three soil types (Fig. 2a, b, and c), indicating a retention of DCD at the soil surface. A greater ($p < 0.001$) percentage of total extractable NH_4^+ in comparison to DMPP-^{14}C was found in the 9-12 cm depth fraction of the sandy loam columns (Fig. 2d). Greater ($p < 0.001$) amounts of total extractable NH_4^+ in comparison to DCD-^{14}C were also found in the 9-12 cm depth fraction of the sandy clay loam columns (Fig. 2e), indicating some dis-location of NI with NH_4^+ at depth. No differences were observed at any depth fraction for DMPP-^{14}C and urine-NH_4^+ in the sandy clay loam or the Sapric Histosol columns (Fig. 2e and f, respectively), indicating similar vertical distributions under conditions of mass flow.

5.3.3 Solubility of DCD and DMPP in water

The solubility of DMPP at 20°C was significantly higher ($p < 0.001$) at 125 ± 2.4 g l^{-1} in comparison to that of DCD at 73.2 ± 2.0 g l^{-1}. An increasing trend was not observed in the

replicates maintained for 48 and 72 h, indicating that saturation of the NI within the matrix had occurred after 24 h.

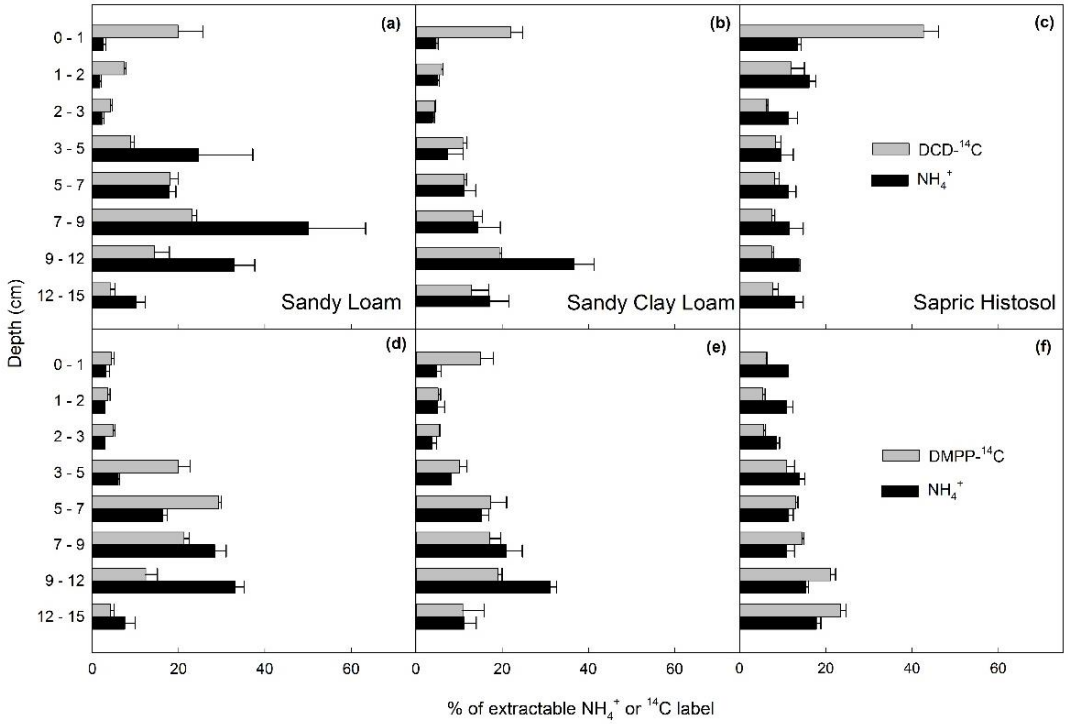

Figure 5.2 The percentage of extractable DCD-^{14}C (a, b and c) or DMPP-^{14}C (d, e and f) compared to NH_4^+ at differing soil depth fractions following a simualted urine deposition plus 40 mm rainfall event applied to sandy loam (a and d), sandy clay loam (b and d) and Sapric Histosol (c and f) columns. Soil type labels apply to each column and legends apply to each row of panels. Bars represent means (n = 3) and error bars denote SEM.

5.3.4 Sorption

Sorption isotherms for DCD-^{14}C (Fig. 3a, c and e), DMPP-^{14}C (Fig. 3b, d and f) and NH_4^+ (Fig. 4) were linear, where all R^2 values were greater than 0.95. The gradient of the linear sorption isotherms were steeper ($p < 0.001$) in the Sapric Histosol compared to the other soil types for both DCD-^{14}C, DMPP-^{14}C and NH_4^+ indicating greater amounts of sorption in this

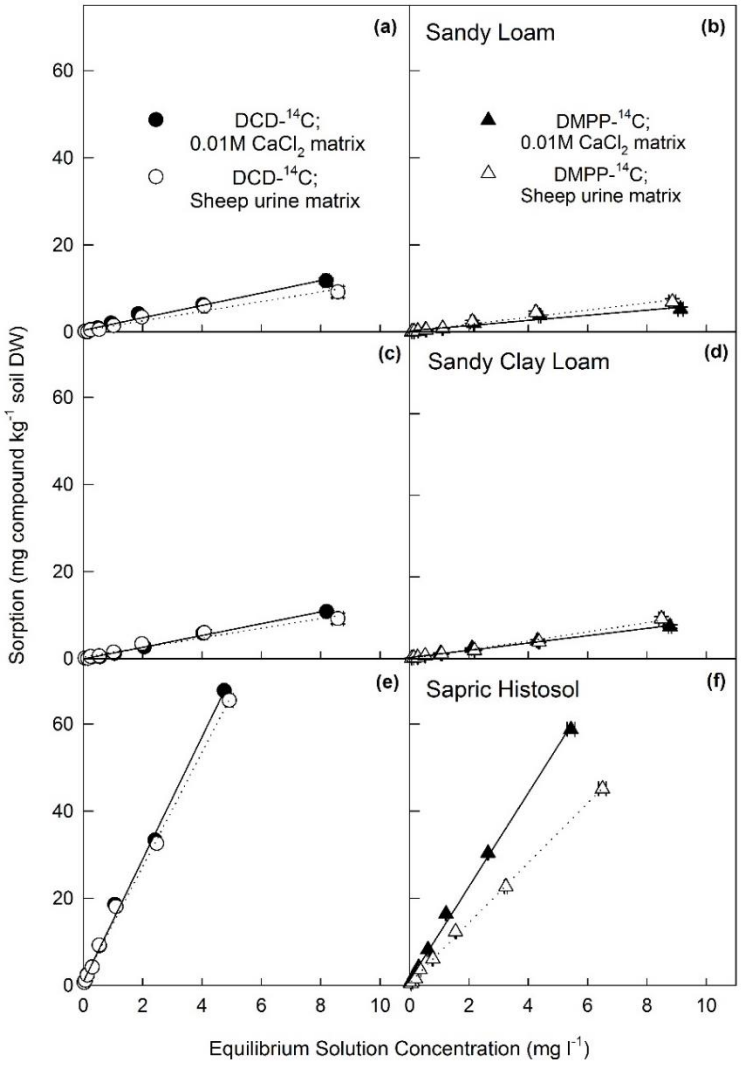

Figure 5.3 Linear sorption isotherms for ^{14}C-DCD (a, c and e) and ^{14}C-DMPP (b, d and f) in either a 0.01 M $CaCl_2$ or sheep urine matrix, in a sandy loam (a and b), sandy clay loam (c and d) and Sapric Histosol (e and f). Symbols represents means (n = 3), bi-directional error bars represent SEM for sorption and equilibrium solution concentrations, legends apply to each column of panels and soil type labels apply to each row of panels.

soil type. In comparison to DMPP-^{14}C, greater sorption occurred for DCD-^{14}C in the Sapric Histosol in both matrices (0.01 M $CaCl_2$ and urine). However, no differences were observed between DCD-^{14}C and DMPP-^{14}C sorption in the other two soil types ($p > 0.05$). In the Sapric Histosol the gradient of the DMPP-^{14}C sorption isotherm was steeper ($p < 0.001$) in the 0.01

M $CaCl_2$ matrix as opposed to the urine matrix. The calculated soil-to-solution partition coefficients (K_d; Table 2) followed the trend sandy loam < sandy clay loam < Sapric Histosol for DCD-^{14}C, DMPP-^{14}C and NH_4^+; for the NI this trend was observed at both concentrations and within both matrices.

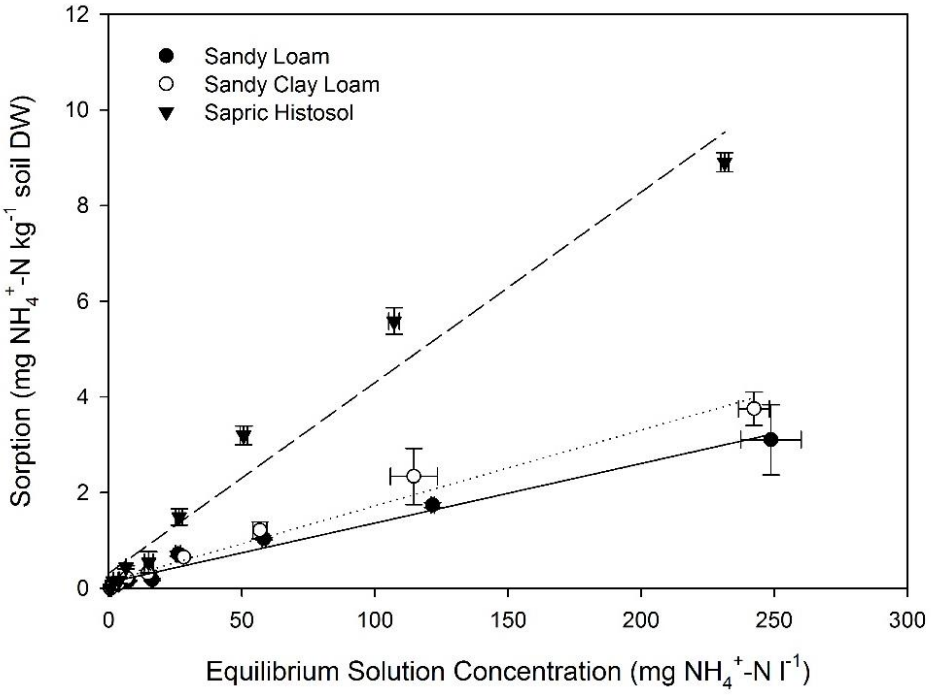

Figure 5.4 Linear sorption isotherms of NH_4^+ in 0.01 M $CaCl_2$ matrix, in three soils (sandy loam and sandy clay loam textured Eutric Cambisol and a Sapric Histosol). Symbols represents means (n = 3) and bi-directional error bars represent SEM for sorption and equilibrium solution concentrations.

Table 5.2 Soil-to-solution partition coefficient (K_d) for the nitrification inhibitors DCD and DMPP at field relevant concentrations (1 and 10 mg l^{-1}) with and without the presence of sheep urine. Values represent means (n = 3) ± SEM.

Soil Type	Matrix	DCD		DMPP		NH_4^+
		1 mg l^{-1}	10 mg l^{-1}	1 mg l^{-1}	10 mg l^{-1}	300 mg N l^{-1}
Sandy loam textured Eutric Cambisol	0.01 M $CaCl_2$	1.25 ± 0.10	1.34 ± 0.05	0.87 ± 0.01	0.63 ± 0.05	1.27 ± 0.35
Sandy clay loam textured Eutric Cambisol	0.01 M $CaCl_2$	1.88 ± 0.05	1.48 ± 0.09	1.15 ± 0.05	0.93 ± 0.05	1.56 ± 0.18
Sapric Histosol	0.01 M $CaCl_2$	15.6 ± 0.28	14.3 ± 0.31	12.8 ± 0.33	11.0 ± 0.44	3.86 ± 0.11
Sandy loam textured Eutric Cambisol	Sheep urine	0.64 ± 0.10	0.64 ± 0.18	0.96 ± 0.05	0.84 ± 0.08	-
Sandy clay loam textured Eutric Cambisol	Sheep urine	1.47 ± 0.15	1.17 ± 0.18	1.01 ± 0.04	1.11 ± 0.07	-
Sapric Histosol	Sheep urine	15.0 ± 0.36	13.4 ± 0.66	7.73 ± 0.43	6.99 ± 0.35	-

5.3.4 Desorption

Generally, the presence of urine increased total desorption (Fig. 5) of both DCD-^{14}C and DMPP-^{14}C numerically (although not statistically) at the fourth consecutive wash, in all soil types. The presence of urine increased desorption of DMPP-^{14}C in the Sapric Histosol at 1 mg l^{-1} ($p < 0.001$; Fig. 5k) and 10 mg l^{-1} ($p < 0.05$; Fig. 5l). Interestingly, the same trend was not observed for DCD-^{14}C in the same soil type (Fig. 5i and j). Desorption of DCD-^{14}C in the $CaCl_2$ matrix was greater ($p < 0.05$) in the sandy loam soil (Fig. 5a and b) compared to the

Sapric Histosol (Fig. 5i and j), but desorption was no greater ($p > 0.05$) in the sandy clay loam soil (Fig 5e and f). In the urine matrix desorption of DCD-^{14}C was lower ($p < 0.01$) in the Sapric Histosol (Fig. 5i and j) compared to the sandy loam (Fig. 5a and b) and sandy clay loam (Fig. 5e and f) textured Eutric Cambisol at both studied concentrations. For both studied concentrations of DMPP-^{14}C in the $CaCl_2$ matrix, desorption was lower ($p < 0.01$) in the Sapric Histosol (Fig. 5k and l) in comparison to either the sandy loam (Fig. 5c and d) or the sandy clay loam (Fig. 5g and h) Eutric Cambisol. In the urine matrix, however, no differences in desorption of DMPP-^{14}C was observed at either concentration between the soil types.

When comparing between the applied ^{14}C-NI in the $CaCl_2$ matrix, there was a greater desorption ($p < 0.05$) of DMPP-^{14}C (Fig. 5e and f) in comparison to DCD-^{14}C (Fig. 5g and h) in the sandy clay loam. The same trend was true for the urine matrix, except no differences were observed between DCD-^{14}C and DMPP-^{14}C at 1 mg compound l^{-1}. In the urine matrix, greater ($p < 0.001$) amounts of DMPP-^{14}C (Fig. 5k and l) desorbed in comparison to DCD-^{14}C (Fig. 5i and j) in the Sapric Histosol, at both studied concentrations. The final wash conducted with 0.5 M K_2SO_4 typically only increased desorption by minor amounts (ranging from 1.4 to 6.0%), indicating that the previous washes had removed the majority of the extractable NI-^{14}C from the soils. The mass balance for the total recovered DCD-^{14}C and DMPP-^{14}C following biological oxidation was 101 ± 0.63% and 101 ± 0.95% respectively, for all soil types, applied concentrations and matrices.

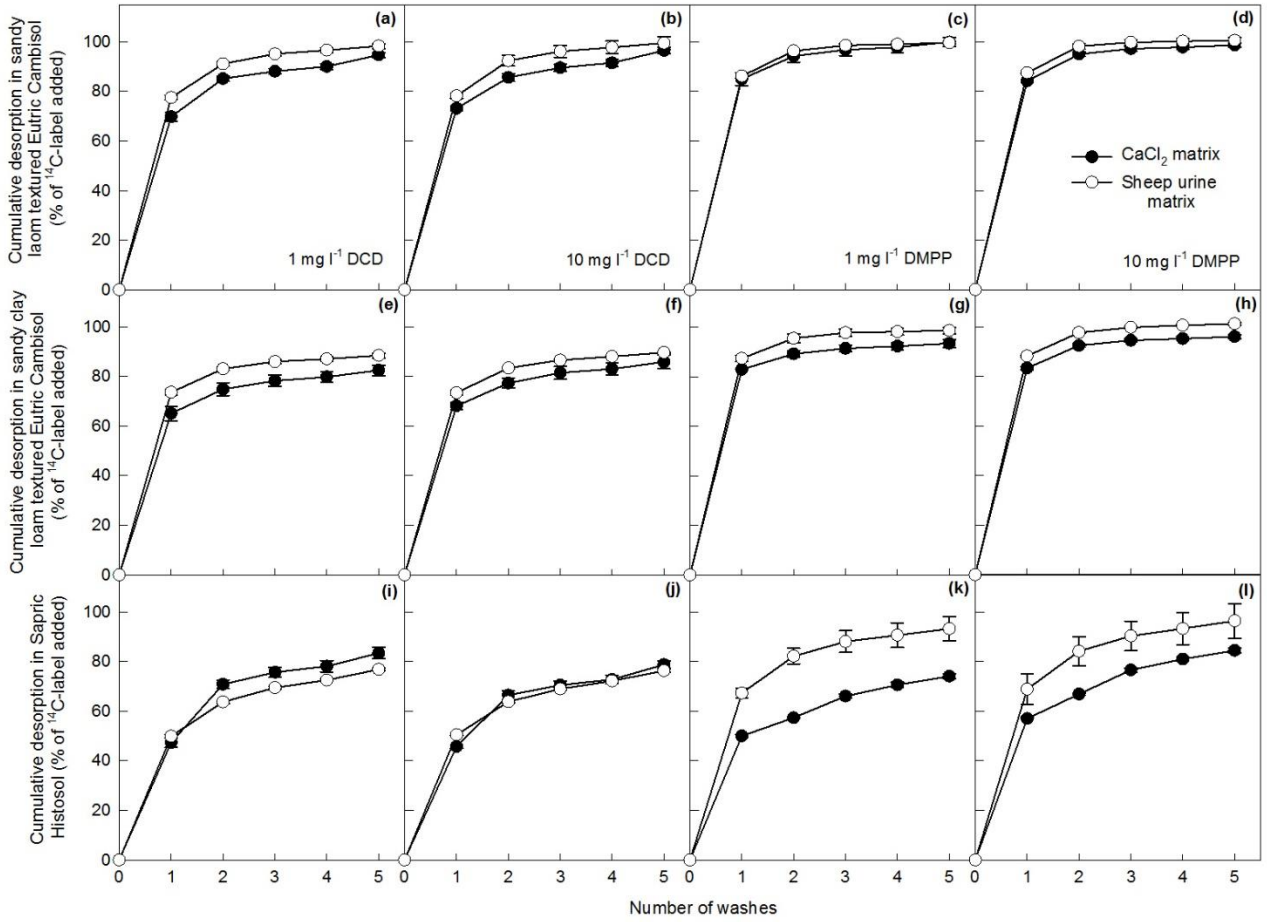

Figure 5.5 Cumulative desorption of ^{14}C-DCD and ^{14}C-DMPP in a sandy loam (a, b, c and d), sandy clay loam (e, f, g and h) and a Sapric Histosol (i, j, k and l) at 1 mg DCD l^{-1} (a, e and i), 10 mg DCD l^{-1} (b, f and j), 1 mg DMPP l^{-1} (c, g and k) and 10 mg DMPP l^{-1} (d, h and l) in either a 0.01 M $CaCl_2$ or sheep urine matrix. Text in the top row of panels applies to each respective column of panels and legend applies to all panels. Symbols represents means (n = 3) and error bars represent SEM for sorption and equilibrium solution concentration.

5.3.5 Mineralization

The results of the mineralization assay confirmed that only minor degradation of the ^{14}C-NI would have occurred under the conditions and duration (0.5 h) of the column study. Mineralization of ^{14}C-DCD within all three soil types ranged from 0.10 to 0.35% of added ^{14}C label, over 0.5 h. For ^{14}C-DMPP the amount degraded over the period of the column incubation was even lower, ranging from 0.03 to 0.16% of the added ^{14}C label. After 8 h, the amount of ^{14}C-DCD mineralized was still low, ranging from 0.41 to 1.67% of the ^{14}C-label applied in the three soil types and at both studied concentrations; the amount of ^{14}C-DMPP mineralized after 8 h ranged from 0.05 to 0.25% of the applied label. Greater amounts ($p < 0.01$; 0.84 ± 0.15 and 1.53 ± 0.30% more at 0.1 and 1 g NI l^{-1}, respectively) of ^{14}C-DCD mineralized in the Sapric Histosol in comparison to ^{14}C-DMPP, at both studied concentrations. The same pattern was also seen for the sandy clay loam textured Eutric Cambisol at 0.1 g NI l^{-1}, where 0.83 ± 0.12% more ^{14}C-DCD was mineralized in comparison to ^{14}C-DMPP. No differences were observed in the amount of ^{14}C-DMPP mineralized between all soil types at either studied concentration. For ^{14}C-DCD at 0.1 g l^{-1}, 1.17 ± 0.21% and 0.69 ± 0.18% more^{14}C-DCD mineralized in the Sapric Histosol in comparison to either the sandy loam or sandy clay loam textured Eutric Cambisol, respectively. No differences ($p > 0.05$) were observed in the amount of ^{14}C-DCD mineralized between the different soils at the higher studied concentration.

5.3.6 Microbial uptake

After 1 h in the sandy loam textured Eutric Cambisol, no difference ($p < 0.05$) was observed between the amount of DCD-^{14}C or DMPP-^{14}C acquired by the soil microbes, which ranged between 20 and 23% of that applied, at both studied concentrations. The same trend was observed in the sandy clay loam textured Eutric Cambisol, where uptake ranged from 18 to 28% of that applied. In the Sapric Histosol, greater amounts ($p < 0.001$) of DCD-^{14}C (66 ±

0.36%) was acquired by soil microbes in comparison to DMPP-^{14}C (51 ± 2.67%) at the higher application rate, however, no differences ($p > 0.05$) between DCD-^{14}C (56 ± 0.32%) and DMPP-^{14}C (61 ± 1.01%) were observed at the lower concentration. The microbial uptake was two to three-fold greater ($p < 0.001$) in the Sapric Histosol compared to the mineral soils for both NI and at both studied concentrations. The results of the microbial uptake study correspond well with that of the soil column studies, indicating that the deficit in the amount of ^{14}C-NI recovered from the soil columns is that which was immobilised into microbial biomass and degraded within the soils.

5.4 Discussion

Our first hypothesis was that DCD would be more mobile and translocate further down the soil profile than DMPP, due to the positive charge and rapid sorption of DMPP to soil colloids (Azam et al. 2001). The results of the column study investigating the vertical movement of DCD and DMPP over 0-15 cm under a 40 mm rainfall event, revealed that the mobility of both NI were similar, and DCD did not appear to be more mobile than DMPP. A greater sorption for DCD in comparison to DMPP was found in the organic and mineral soils, contradicting our hypothesis that a greater adsorption of DMPP would occur. A greater sorption was found in the Sapric Histosol compared to the mineral soils for both NI, suggesting negatively charged domains within organic matter are important for adsorption processes. However, if the results of Fig.5 are expressed on a per g of organic C basis, the results between the soils are more similar, suggesting a partition phenomenon rather than charged-based sorption. Compounds possessing a greater octanol-water partition coefficient than others will show slower sorption; the octanol-water partition coefficient (Log P) of DCD and DMPP is predicted to be -1.03 and 0.92 for DCD and DMPP, respectively (Chemicalize.org 2016). In addition, DCD is hydrophilic (Turowski and Deshmukh 2004), which in combination with the

low value for the octanol-water partition coefficient, suggests strong absorption and permeation into organic matter. Protection and occlusion of NI from nitrifiers and other microbes due to sorption, may reduce the effectiveness of NI, at least in the short term. Sorption may also protect against some microbial degradation, and if remobilisation of NI occurs, it may prolong the inhibitory effect (Barth et al. 2001).

Under some circumstances DMPP has been found to be more effective than DCD at inhibiting nitrification and reducing N_2O losses (Weiske et al. 2001; Chaves et al. 2006; Irigoyen et al. 2006) and this difference in efficacy is often attributed to the lower mobility of DMPP in comparison to DCD and hence a greater spatial separation of DCD with NH_4^+. Nevertheless, the inhibition of the oxidation of NH_4^+ to NO_3^- only occurs when the nitrifying population have taken up the NI. This study only examined the mobility of NI and NH_4^+, however, a consideration of the distribution of nitrifying microorganisms and their acquisition of the NI is an important aspect for future research. Our second hypothesis was that DMPP would co-locate with urine-NH_4^+ more than DCD. In our study, both NI appeared to coincide well with urine-derived NH_4^+, with only few incidences of the percentage of extractable NH_4^+ being higher than the extractable NI-^{14}C label at depth. Nevertheless, this study only focused on the short-term co-location of NI with urine-NH_4^+, and further generation of urine-NH_4^+ would occur post urea hydrolysis. Being a neutral compound, urea may also be susceptible to vertical transport (Dawar et al. 2011). However, urea hydrolysis is normally complete within ca. 2 days, reducing the time available for vertical movement. Comparing the soil-to-solution partition coefficients of the NI and NH_4^+, it appears that the K_d of NH_4^+ and DCD are more similar than that of DMPP and NH_4^+ in the sandy loam and the sandy clay loam soils, but large differences were found for both NI compared to NH_4^+ in the Sapric Histosol. It may be possible to use NI and NH_4^+ K_d values from differing soil types in order to assess which (if any) inhibitor may be more effective, where similar K_d values may result in a greater co-location of the two

chemicals. Further work is required to assess whether this would be a useful proxy for reducing N_2O emissions and improving NI use in order to maximise efficacy.

The third objective was to determine if the presence of sheep urine resulted in a greater vertical movement of both NI. Without the presence of urine (rainfall only), a retention of DCD was observed in the top 1 cm of the sandy loam and sandy clay loam textured soils, and a retention of both NI was found in the top 1 cm of the Sapric Histosol. A retention of NI at the surface may be beneficial in that nitrification decreases with pasture soil depth (Young et al. 2002), which may result in a greater coincidence of NI with nitrifiers, nevertheless, the use of sieved soils in this study would have altered any natural depth distribution of nitrifiers in the incubated soil profiles. The addition of sheep urine to the soil columns had a mixed effect on the depth distribution of the NI, depending on the soil type and inhibitor. Relative to simulated rainfall alone, the presence of sheep urine resulted in a greater amount of extractable DCD-^{14}C at the bottom of the sandy loam soil columns, and reduced the extractable amount of both DCD-^{14}C and DMPP-^{14}C in the top 1 cm of the sandy loam soil columns, however, no differences in depth distribution of either NI were observed following sheep urine and rainfall application to the sandy clay loam soil. The presence of sheep urine resulted in lower amounts of DMPP-^{14}C at the top 1 cm and greater amounts of extractable DMPP-^{14}C at 12-15 cm in the Sapric Histosol, but no such trend was observed for DCD-^{14}C. To consider the reasons behind these results, a consideration of the soil, NI and urine properties are required.

The soils used in this study were all of a similar pH (Table 1), however the addition of urine would have altered the soil pH and made conditions in the soil columns more alkaline. As DCD is amphipathic, sorption has been shown to be pH dependent, where increases in pH above pH 5 lead to increased sorption (Zhang et al. 2004). This may partially explain why the vertical distribution of DCD-^{14}C was similar whether urine was present or not in the Sapric Histosol. In the solubility assay, DMPP was found to be over 1.5 times more soluble in water

than DCD. However, a saturated solution (125 g l^{-1}) of DMPP is acidic (ca. pH 3), whereas dissolving DCD results in a near neutral pH. The solubility of DMPP at pH 7, is reported to be 46 g l^{-1} (Zerulla et al. 2001) - considering this value results in DCD having a greater solubility than DMPP. Thus, DMPP solubility may vary widely as a function of soil pH and buffer capacity, but whether this influences the mobility relative to DCD is unclear. As the soils used in this study had a similar pH, NI solubility would not have varied much due to the effect of soil type.

The sandy loam and sandy clay loam soils had a similar CEC and organic matter content (Table 1), however, both parameters were greater in the Sapric Histosol. The NI sorption and partitioning into organic matter, and the availability of cation exchange sites may have all contributed to the differences in the distribution of the NI, with and without the presence of urine. The low CEC in the mineral soils and saturation of these exchange sites with cations present within the urine, may explain the higher amounts of DCD-^{14}C and DMPP-^{14}C at the bottom of the sandy loam soil columns, compared to the rainfall only treatment. This trend did not hold true, however, for the sandy clay loam columns despite the similarity in soil properties.

The results of our desorption assays revealed that even after one wash with 0.01 M $CaCl_2$, a large proportion of DCD and DMPP was remobilised into solution. This supports the theory that remobilisation of these NI may occur e.g. following urine deposition and/or heavy rainfall events. In the case of ruminant urine events this effect may be important, as urine is generally deposited at varying times following NI application to pasture. While the presence of urine generally increased desorption, the effect was strongest for DMPP-^{14}C in the Sapric Histosol, and weakest for DCD-^{14}C in the same soil type. This again shows a clear contrast in the binding mechanisms and behaviour of these two NI. In the Sapric Histosol, the vertical movement of DMPP was enhanced due to the presence of urine. This trend was not observed for DCD, which may be due to differences in the physico-chemical properties of these NI. The

partitioning and adsorption of DCD in the organic soil may have prevented its vertical movement due to urine addition.

The short-term microbial mineralization of DCD was faster in comparison to DMPP in all soil types, which supports our fourth hypothesis. This is consistent with results of other studies, where DMPP has been found to have a longer residence time in comparison to DCD (Chaves et al. 2006). DCD degrades to CO_2 and NH_4^+ via guanilyc urea, guanidine, and urea (Amberger 1986; Kelliher et al. 2008). The first stage of DCD degradation can occur on the surface of metal oxides, which catalyse the reaction of DCD and water to guanylurea (Hallinger et al. 1990). Biological degradation of DCD by common soil microorganisms has also been demonstrated, where DCD is supplied as the sole N source in pure culture (Hauser and Haselwandter 1990; Schwarzer et al. 1998). As DMPP is a heterocyclic compound, it is not readily degradable (Chaves et al. 2006), although information on the mechanism and degradation pathways of this inhibitor are still lacking. Results from this study indicate that the microbial community were better able to degrade DCD in comparison to DMPP, where DMPP degrading bacteria may take longer time periods to establish in comparison to DCD. DMPP has also been found to have a longer effect on the abundance of ammonium oxidizing bacteria in comparison to DCD (Kuo et al. 2015) and there is evidence that DMPP has an inhibitory effect on both ammonium oxidizing bacteria and archaea (Florio et al. 2014).

In this study, even after 1 h, the microbial uptake of both inhibitors accounted for a large proportion of that applied. Approximately 20% was taken up by soil microbes in the mineral soils and > 50% of that applied was taken up by soil microbes in the Sapric Histosol, which was likely to be a function of the greater microbial biomass in this soil. To be effective, the NI would need to be acquired by the target microbial communities (ammonium oxidizing bacteria and archaea). Immobilisation into non-target microbial communities could, therefore, equate to a fairly large removal mechanism for these NI and this requires further investigation.

No difference was observed between DCD and DMPP in the proportion acquired by soil microbes in the mineral soils. However, uptake was greater for DCD compared to DMPP in the Sapric Histosol at the higher studied concentration. This suggests a slight preference of, or bioavailability of DCD to the soil microbial community in the short-term.

The results of this study should be considered with care, as repacked soils were used and there were no preferential flow pathways as would occur under field conditions, which would potentially enhance the vertical movement of either NI. The soils were also sieved, which removed any natural variation of soil properties which can occur with depth. The use of soil columns could have also promoted vertical movement of NI or urine, by restricting lateral diffusion. This is in contrast to the study by Azam et al (2001), where shallow petri dishes were used and NI and NH_4^+ were applied in the centre. This approach may have promoted lateral movement of solutes. The soils used to repack the soil columns in this study were field moist and the initial starting moisture content of the soil could also influence the movement of inhibitors through the soil. Further work should attempt to establish the comparative efficacy of both NI, their uptake by nitrifying and non-nitrifying microorganisms and co-location with NH_4^+ over longer time scales and under field conditions with differing soil moisture contents.

5.5 Conclusions

A similar distribution of DCD and DMPP was observed up to a depth of 15 cm following a simulated rainfall event in one organic and two mineral soils. The presence of sheep urine did not influence the depth distribution of DCD following rainfall, but enhanced the movement of DMPP down the profile, especially in the organic soil. A greater sorption was found for DCD in comparison to DMPP in the soil types studied here and the presence of urine generally increased desorption of both NI. The results of our study suggest that the efficacy of

NI are influenced more by differences in microbial uptake and degradation rates than by differences in sorption and desorption rates to the soil matrix.

5.6 Acknowledgements

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Chapter 6

Plant acquisition and metabolism of the synthetic nitrification inhibitor dicyandiamide and naturally-occurring guanidine from agricultural soils

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KAM, MS, PWH, DLJ and DRC designed and conceived the experiment; KAM conducted the practical work; KAM analysed the results and prepared the manuscript.

Abstract

There is increasing interest and use of nitrification inhibitors (NI) in agroecosystems, yet little is known of their fate *in planta*. Residues of the organic, N-rich NI, dicyandiamide (DCD), have been found in milk products following commercial application to pasture. We investigated whether plant acquisition and metabolism of DCD were consistent with plant-mediated transmission from soil to agricultural food products. Uptake rates, translocation to the shoot, degradation of the label within wheat tissue and availability within two soils of DCD and the structurally similar naturally occurring N-rich molecule, guanidine, were measured using ^{14}C labelling. Under sterile conditions, over 2 h wheat took up (34 and 14 µmol g^{-1} root DW h^{-1} at 1 mM: DCD and guanidine, respectively), translocated (7-15 and 19-22%) and metabolised (0.4 and 0.9% of uptake) DCD- and guanidine-^{14}C. Both molecules were also acquired from soil by wheat despite concurrent soil sorption and microbial uptake. Both DCD and guanidine can be acquired and metabolised by graminaceous plants. Although probably not a significant route of N acquisition, plant uptake provides a direct route of DCD entry into the food chain.

Key Words: Bioavailability; Dicyandiamide (DCD); Guanidine; Mineralization; Nitrification inhibitor; Nitrogen cycle.

6.1 Introduction

Within agricultural soils, nitrification represents one of the dominant nitrogen (N) flow pathways and is responsible for generating NO_3^- which can be lost to the wider environment via leaching and denitrification (Zerulla et al. 2001). To reduce these N losses, effective management strategies are required to improve N use efficiency (NUE) within most agroecosystems. One potential solution to the problem is the application of synthetic or natural nitrification inhibitors (NI) to the soil to slow the conversion of NH_4^+ to NO_3^- (Subbarao et al. 2012; Abalos et al. 2014).

Among the many identified NI, synthetic dicyandiamide (DCD; $C_2H_4N_4$) is one of the most widely researched and one of the few used at a commercial scale (O'Callaghan et al. 2010; Liu et al. 2013). DCD has been investigated in a wide range of arable and livestock-based agroecosystems, where applications of DCD (10-30 kg ha^{-1}) have been shown to be effective in reducing nitrous oxide (N_2O) emissions following spreading of either N fertilisers (Weiske et al. 2001; Di and Cameron 2006; Cui et al. 2011), livestock slurry (Hatch et al. 2005) or ruminant urine (Di and Cameron 2006; Dai et al. 2013; Barneze et al. 2015). DCD application to soil has also been shown to reduce NO_3^- leaching after the application of inorganic N fertilisers and from livestock urine patches (Di et al. 2009; Cui et al. 2011). A recent meta-analysis investigating the effect of soil-applied NI (including DCD), indicated that, on average, they result in an 8% increase in crop yield and a 13% increase in NUE (Abalos et al. 2014).

A range of application routes for DCD have been investigated including introduction to urine patches by oral administration to cattle (O'Connor et al. 2013; Welten et al. 2013), infusion of DCD into the rumen or abomasum of sheep (Ledgard et al. 2008), incorporation into fertiliser granules or addition of DCD in a biodegradable hydrogel to slow its release in soil (Minet et al. 2013). However, the simplest, least controversial and consequently most

widespread route is direct application to soil. Although direct application to soil is both practical and has demonstrable efficacy, this method leaves DCD susceptible to degradation by soil microbes, which may reduce persistence and increases in NUE. There is also the potential for removal from soil due to uptake by plant roots.

In 2012, low level residues of DCD were found in New Zealand dairy products from which plant interception and uptake of DCD with subsequent transfer to ruminant milk have been hypothesised to be key vectors in its contamination (Kim et al. 2012; Chen et al. 2014). Although DCD is not currently known to pose a significant risk to human health, the discovery of DCD in milk has led to a voluntary suspension of sale and use of DCD in New Zealand until international acceptable limits for its presence in milk products can be agreed (Ministry for Primary Industries 2013).

Whether plants are able to take up DCD is currently uncertain. Similarly, whether (if taken up) DCD persists in plant tissues or is rapidly degraded is unknown. Historically, DCD has been suggested for use as an organic fertiliser, although as opposed to intact plant uptake of this molecule, prior degradation to NH_4^+ (and subsequent nitrification to NO_3^-) was the presumed route of N supply to plants (Reddy 1964). Limited evidence from some >100 year old investigations (Hutchinson and Miller 1912) show increased soil residence of DCD in the absence of plants, and reported phytotoxicity and yield reduction at high rates of DCD addition (25 kg ha^{-1}) to clover, suggest that plant uptake does take place (Amberger 1986; Di and Cameron 2004; Macadam et al. 2003; Kelliher et al. 2008). The occurrence of the structurally similar molecule guanidine (CH_5N_3; Fig. 6.1) and derivatives in a wide range of organisms including soil, plants and associated microbes may also suggest the existence of capacity for acquisition and assimilation of DCD by plants (Kato et al. 1986; Prescott and John 1996; Schulten and Schnitzer 1998; Kawano and Hwang 2010; Güthner et al. 2014).

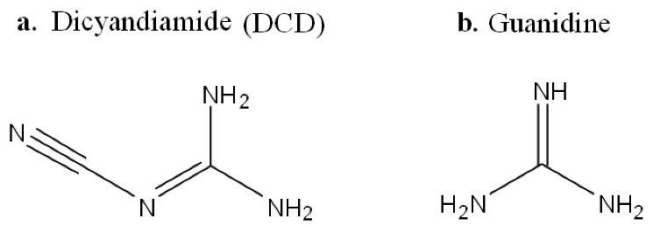

Figure 6.1 Molecular structure of the nitrification inhibitor, dicyandiamide (DCD; Panel A), and its naturally occurring analogue guanidine (Panel B), which are used in this study.

This investigation was predicated on the need to determine whether 1) the capacity of plants to take up DCD through their roots and translocate it to shoots is great enough, and 2) that the rate of subsequent degradation *in planta* is slow enough, to make plants a realistic route for the transmission of DCD from soil to agricultural food products. It also presents an opportunity to investigate earlier speculation on the potential of guanidine and similar compounds to be added to the rapidly growing list of routes via which plants have the capacity to obtain N from soil (Hutchinson and Miller 1912; Lewis 1936; Bollard 1966; Warren 2014).

We aimed to test the following hypotheses: 1) graminaceous plants have the capacity to take up both DCD and guanidine with their roots; 2) both DCD and guanidine can be metabolised by plants; 3) plants can take up both DCD and guanidine from soil; 4) the magnitude of competing substrate removal processes from soil (microbial uptake, mineralization and sorption) will be regulated by soil type.

6.2 Materials and methods

6.2.1 Soil properties

Two contrasting UK agricultural soils were used in this study (Table 6.1). The first was a mineral sandy loam textured Eutric Cambisol collected from a sheep-grazed fertilised grassland in North Wales (53°14′N, 4°01′W), while the second was an organic Sapric Histosol

Table 6.1 Soil properties of Eutric Cambisol and Sapric Histosol used in soil rhizosphere microcosms. Values represent means ± SEM, *n* = 4, letters indicate significant differences between the two soils and results are reported on a dry soil weight basis.

Soil Property	**Eutric Cambisol**	**Sapric Histosol**
Moisture Content (%)	25.0 ± 0.27 a	61.2 ± 0.09 b
Organic Matter (%)	7.70 ± 1.41 a	77.2 ± 0.47 b
Cation Exchange Capacity (meq 100 g^{-1})	14.8 ± 0.68* a	80.8 ± 0.80* b
pH	6.67 ± 0.14 a	6.37 ± 0.06 a
Electrical Conductivity (µS cm^{-1})	50.1 ± 1.85 a	103 ± 9.56 b
Total Carbon (g kg^{-1})	23.8 ± 1.50 a	391 ± 1.15 b
Total Nitrogen (g kg^{-1})	2.92 ± 0.07 a	26.5 ± 0.07 b
Total Organic Carbon (mg C kg^{-1})	88.2 ± 6.29 a	959 ± 79.1 b
Total Organic Nitrogen (mg N kg^{-1})	10.6 ± 3.70 a	37.8 ± 5.35 b
Microbial C (g kg^{-1})	1.58 ± 0.05 a	4.41 ± 0.20 b
Microbial N (g kg^{-1})	0.33 ± 0.03 a	0.96 ± 0.08 b
NH_4^+ (mg N kg^{-1})	4.29 ± 0.32 a	5.90 ± 0.69 a
NO_3^- (mg N kg^{-1})	4.34 ± 0.20 a	27.8 ± 2.45 b
PO_4^{3-} (mg P kg^{-1})	9.98 ± 0.39 a	32.0 ± 6.68 b
K^+ (meq kg^{-1})	12.3 ± 2.36 a	8.46 ± 2.72 a
Na^+(meq kg^{-1})	0.95 ± 0.05 a	5.08 ± 0.37 b
Ca^{2+}(meq kg^{-1})	71.5 ± 11.0 a	619 ± 12.5 b

* *n* = 3

collected from an intensive arable production area in East Anglia (52°52′N, 0°47′W). At each site, four independent replicate soil samples (0-10 cm) were collected, sieved to pass 2 mm, and stored at 4ºC in gas permeable polythene bags until the start of the experiment, where field

moist soils were utilised for experimentation. Soil moisture content was determined by oven drying (105ºC, 24 h), and soil organic matter content by loss-on-ignition (450ºC, 16 h; Ball, 1964). Total soil C and N content was determined using a CHN2000 Analyzer (Leco Corp., St. Joseph, MI). Soil pH and electrical conductivity (EC) were measured using standard electrodes in 1:2.5 (w/v) soil: distilled water extracts. Available C and N was determined using a 1:5 (w/v) soil-to-0.5 M K_2SO_4 extract within 24 h of sample collection, according to Jones and Willett (2006). Total dissolved C and N in the extracts were determined with a Multi N/C 2100S (AnalytikJena, Jena, Germany). Microbial biomass C and N were determined by $CHCl_3$ fumigation-extraction according to Voroney et al. (2008) using K_{EC} and K_{EN} correction factors of 0.35 and 0.50, respectively. K_2SO_4-extractable P, NO_3^- and NH_4^+ were determined using the colorimetric methods of Murphy and Riley (1962), Miranda et al. (2001) and Mulvaney (1996), respectively. Exchangeable cations were measured within 1:5 w/v soil-to-1 M NH_4Cl extracts using a model 410 flame photometer (Sherwood Scientific Ltd., Cambridge, UK).

6.2.2 Plant uptake and translocation of DCD and guanidine under sterile conditions

DCD and guanidine uptake rates were determined under sterile conditions to determine if they were taken up intact (i.e. without prior microbial cleavage). Wheat seeds (*Triticum aestivum* var. Granary) were surface sterilised with 14% (v/v) NaClO and 80% ethanol and grown aseptically according to Hill et al. (2011). Briefly, surface sterilised seeds were germinated on agar containing 50% Murishage and Skoog basal medium, to screen for microbial contamination; after which they were transferred aseptically to Phytatrays (Sigma Aldrich, Gillingham, UK) containing sterile perlite and 50% Murishage and Skoog basal medium, supplemented with 10 mg l^{-1} Na-metasilicate. Wheat plants were grown at 20ºC, with a 16 h photoperiod and light intensity (PAR) of 500 µmol m^{-2} s^{-1}, until they had reached the third leaf stage. The roots of individual intact wheat plants (n = 4) were placed in 12 ml of

sterile (0.2 µm-filtered) solution containing either ^{14}C-DCD or ^{14}C-guanidine (ca. 1 kBq ml^{-1}; American radiolabelled Chemicals, St Louis, MO, USA), for a period of 2 h. A solution concentration of 1 mM DCD was chosen to reflect the DCD concentration found within soil solution in response to typical field application rates (10 kg ha^{-1}) and two lower concentrations of 0.01 and 0.1 mM, were chosen to represent subsequent dilution of field applied DCD by diffusive and mass flow processes. Subsequently, the plant roots were thoroughly rinsed in 0.01 M $CaCl_2$, followed by deionised water and the roots and shoots oven-dried (80ºC, 24 h). To quantify the ^{14}C content of the plants, the roots and shoots were separately combusted in an OX400 biological oxidizer (RJ Harvey, Hillsdale, NJ, USA), the $^{14}CO_2$ captured in Oxosol scintillant (National Diagnostics, Atlanta, GA, USA) and ^{14}C measured using a Wallac 1404 Liquid Scintillation Counter (Wallac EG&G, Milton Keynes, UK). To visualise the location of ^{14}C-DCD and ^{14}C-guanidine in the root and shoot tissues, the ^{14}C distribution within intact plants was imaged using a Cyclone Plus phosphor-imaging system (PerkinElmer, Waltham, MA, USA) using an exposure time of 1 h.

6.2.3 Plant metabolism of DCD and guanidine

To determine whether DCD or guanidine could be mineralized within the plant, sterile wheat seeds were prepared as described previously. The roots of intact plants were then placed in sterile (0.2 µm-filtered) solutions containing either ^{14}C-DCD or ^{14}C-guanidine (4 ml; 1 kBq ml^{-1}; 0.01 mM). The plants were then placed in sterile 250 cm^3 polypropylene vessels through which moist air was passed at a rate of ca. 600 ml min^{-1}. The outflow was bubbled through Oxosol scintillant to capture any respired $^{14}CO_2$. The Oxosol was changed after 1, 5, 10, 20, 40, 60, 90 and 120 min and captured ^{14}C measured as described above.

6.2.4 Plant uptake of DCD and guanidine from soil

To determine the uptake rates of DCD and guanidine from soil, wheat seeds (n = 4) were individually sown into Eutric Cambisol or Sapric Histosol rhizosphere microcosms (240 mm long; internal diameter 8 mm) as described in Owen and Jones (2001). Each microcosm contained approximately 12 g FW soil. Plants were grown under the same conditions used for the sterile uptake study (Section 2.2), until they had reached the third leaf stage. At this point, solutions of ^{14}C-DCD or ^{14}C-guanidine (ca. 1 kBq ml^{-1}; 0.01, 0.1 and 1 mM) were injected directly into the rhizosphere soil. A total of 4 injections (0.25 ml each) using 1 ml polypropylene syringes and 18 gauge needles at four depths (3, 9, 15 and 21 cm) were made, to facilitate an even distribution of solution within the microcosms. After 2 h, plants were removed from the microcosms and washed thoroughly in 0.01 mM $CaCl_2$, followed by distilled water. After drying (80ºC, 24 h), the ^{14}C content of the root and shoot material was determined as described above. To estimate the quantity of root in contact with the injected solution, blue ink was injected as above into another set of microcosms (n = 4). The sections of root exposed to the ink were removed, washed, dried (80 °C) and weighed.

This experiment was repeated under similar conditions, utilising unlabelled DCD (1 mM), in order to establish whether the intact DCD molecule could be detected in wheat shoot extracts via HPLC. After injection of the substrate and washing of the root material, the shoot was separated from the root. The shoots of four wheat plants were bulked (n = 3) in order to increase the likelihood of ascertaining a measurable peak on the HPLC, and ground in 2 ml of DMSO in a borosilicate Griffiths tube; 1 ml of the DMSO extract was then evaporated to dryness under vacuum in a rotary evaporator. The pellet was resuspended in 0.5 ml of HPLC grade water, centrifuged (10 000 g), and analysed for DCD using an adapted method of Turowski and Deshmukh (2004) on a Varian ProStar HPLC, with UV detection at 215 nm. The column used was a Luna 5u SCX (250 x 4.6 mm; 5µm; 100 Å), DCD eluted after ca. four

minutes in this system. Chromatograms were compared to standards and the control plants (no injected DCD).

6.2.5 DCD and guanidine mineralization within soils

DCD and guanidine mineralization were determined, over the same time course as the rhizosphere uptake study (Section 2.5), by measuring the rate of $^{14}CO_2$ evolution after the addition of 0.2 ml of ^{14}C-DCD or ^{14}C-guanidine (ca. 5 kBq ml^{-1}; 0.01, 0.1 and 1 mM) to 1 cm^3 of each soil (n = 4). Soils were contained in a 10 cm^3 sealed glass vessel, with moist air flowing (ca. 100 ml min^{-1}) over the soil surface. Evolved $^{14}CO_2$ was captured by passing the outflow through two consecutive 0.1 M NaOH traps (capture efficiency > 95%; Hill et al. 2007). Traps were changed after 1, 5, 10, 20, 40, 60, 90 and 120 min, and the activity in the solution determined by liquid scintillation counting after mixing with HiSafe 3 scintillant (PerkinElmer, Llantrisant, UK).

6.2.6 DCD and guanidine microbial uptake and sorption within soils

The amount of substrate remaining in soil solution (i.e. that remaining after microbial uptake and abiotic removal processes) was determined according to the centrifugal-drainage procedure of Hill et al. (2008). Briefly, ^{14}C-DCD or ^{14}C-guanidine (0.2 ml; ca. 5 kBq ml^{-1}; 0.01, 0.1 or 1 mM) was pipetted evenly onto the soil surface. After 1, 5, 10, 20, 40, 60, 90 and 120 min, soil solution was recovered by centrifugation (4000 *g*, 1 min, 20ºC) and the amount of ^{14}C-DCD or ^{14}C-guanidine in the recovered soil solution determined by liquid scintillation counting as described above.

The amount of DCD or guanidine sorbed to the solid phase and present in soil solution was determined by performing 0.5 M K_2SO_4 extracts over time. Briefly, ^{14}C-DCD or ^{14}C-guanidine was mixed with soil and incubated as described above. After 5, 10, 20, 40, 60, 90 or

120 min the soil was extracted with ice-cold 0.5 M K_2SO_4 (1:5 w/v) by shaking (150 rev min^{-1}) for 20 min. After shaking, the extracts were centrifuged (10,000 *g*, 10 min) and ^{14}C in the supernatant determined by liquid scintillation counting. The amount of substrate sorbed to the soils was calculated by deducting the soil solution pool from the K_2SO_4 extractable pool; the amount of substrate taken up by soil microbes was determined by deducting the soil solution pool and the sorbed pool from the total substrate added.

6.2.7 Statistical analysis

All measurements were performed in quadruplicate unless otherwise stated. Log-transformation of data, followed by a one-way ANOVA with Fisher's LSD post-hoc test, was used to compare the following: wheat root uptake rates of DCD and guanidine under sterile conditions, the percentage of substrate translocation to wheat shoot biomass under sterile conditions, wheat root uptake rates from soil rhizosphere microcosms (after correcting for root weight exposed to injected solutions), percentage of substrate translocation to shoot biomass from wheat grown in soil, microbial uptake and sorption of the substrate at the 2 h time point. Data were tested for normality (Shapiro-Wilk test) and homogeneity of variance assumptions (Levene's test) prior to conducting the ANOVA. Microbial mineralization data were analysed by one-way ANOVA as above, except data were not transformed. An independent samples t-test was used to compare plant metabolism of assimilated compounds at the 2 h time point, after testing the normality and homogeneity of variance assumptions. All statistical analyses were performed in IBM SPSS Statistics 20.0.

6.3 Results

6.3.1 DCD and guanidine uptake and tissue localization in sterile wheat plants

Rates of uptake of ^{14}C-DCD and ^{14}C-guanidine by wheat roots increased ($p < 0.001$) with increasing concentration (from 0.01 to 1 mM) in sterile solution (Table 6.2; Fig. 6.2). Phosphorimaging revealed a fairly even distribution of the ^{14}C label added as DCD within the shoot biomass (Table 6.2; Fig. 6.3), with the majority remaining in the roots. Phosphorimaging of ^{14}C added as guanidine in the wheat plant showed an even distribution throughout the root system, but it was present predominantly in the lower regions of the shoot (Table 6.2; Fig.6.3). Wheat roots acquired 2.37% less ($p < 0.01$) of the ^{14}C label added as DCD in comparison to guanidine at a concentration of 0.01 mM. No differences ($p > 0.05$) were observed at 0.1 mM, while 1.71% more ($p < 0.01$) DCD was acquired in comparison to guanidine at 1 mM.

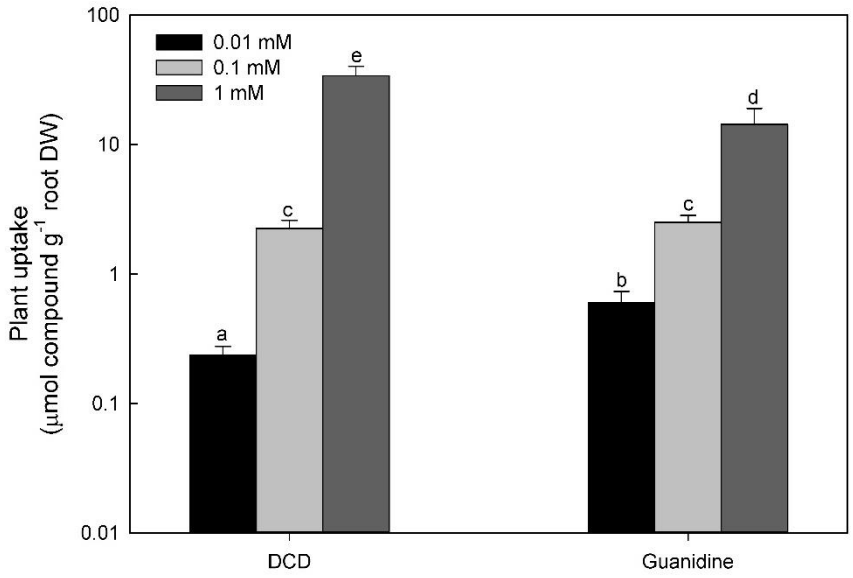

Figure 6.2 Rates of ^{14}C-dicyandiamide (DCD) and ^{14}C-guanidine uptake by wheat (*Triticum aestivum*) roots grown in sterile hydroponic culture at three concentrations (0.01, 0.1 and 1 mM). Values represent means ± SEM ($n = 4$) and different letters indicate significant differences between means of compounds and concentrations (Fisher's LSD; $p < 0.05$).

Table 6.2 Summary of results for wheat uptake rates and tissue localization of ^{14}C-DCD and ^{14}C-guanidine (0.01, 0.1 and 1 mM) under sterile conditions and from Eutric Cambisol and Sapric Histosol rhizosphere microcosms following a 2 h chase period. Values represent means ± SEM (n = 4).

	Experimental condition	**^{14}C-DCD**			**^{14}C-Guanidine**		
		0.01 mM	**0.1 mM**	**1 mM**	**0.01 mM**	**0.1 mM**	**1 mM**
Uptake rate (µmol g^{-1} root DW h^{-1})	Sterile	0.23 ± 0.04	2.25 ± 0.35	34.0 ± 6.29	0.60 ± 0.13	2.51 ± 0.32	14.3 ± 4.72
Uptake rate (nmol g^{-1} root DW h^{-1})	Eutric Cambisol	3.02 ± 0.92	30.9 ± 6.17	353 ± 94.3	7.13 ± 1.88	49.8 ± 13.9	328 ± 66.0
Uptake rate (nmol g^{-1} root DW h^{-1})	Sapric Histosol	2.18 ± 0.57	21.1 ± 1.72	174 ± 13.4	5.25 ± 0.77	47.2 ± 14.7	211 ± 39.2
Shoot translocation (% of acquired label)	Sterile	7.46 ± 1.18	7.76 ± 0.50	15.0 ± 4.05	22.4 ± 3.96	19.0 ± 6.58	22.0 ± 3.18
Shoot translocation (% of acquired label)	Eutric Cambisol	9.13 ± 2.28	5.42 ± 0.51	7.37 ± 2.36	0.71 ± 0.25	1.58 ± 0.40	2.09 ± 0.48
Shoot translocation (% of acquired label)	Sapric Histosol	7.80 ± 1.72	7.55 ± 1.48	7.16 ± 1.54	0.94 ± 0.13	1.10 ± 0.64	2.24 ± 0.20

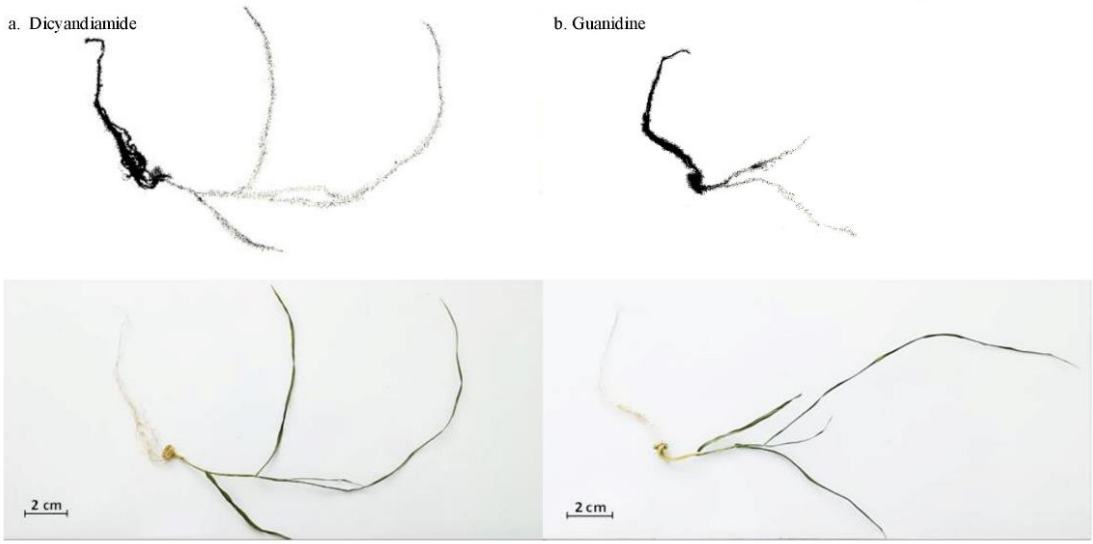

Figure 6.3 Phosphorimages showing the distribution of ^{14}C-dicyandiamide (DCD; Panel A) and ^{14}C-guanidine (Panel B) within sterile wheat plants. The plants were exposed to each substrate (0.01 mM) for 2 h prior to imaging. The dark colour in the top images represents the distribution of ^{14}C-label within the plant tissue while, the images underneath are the corresponding photographs of the same plants.

6.3.2 Plant mineralization of assimilated DCD and guanidine

Of the added ^{14}C-DCD and ^{14}C-guanidine taken up by the plant, only small amounts were mineralized to $^{14}CO_2$ during the experiment. After 2 h, 0.44 ± 0.07% of the acquired ^{14}C-DCD and 0.90 ± 0.22% of acquired ^{14}C-guanidine had been metabolised to $^{14}CO_2$ within the plant with no differences observed between the two substrates ($p > 0.05$). For both compounds, respiration rates tended to be faster in the first 20 minutes of the incubation (Fig. 6.4).

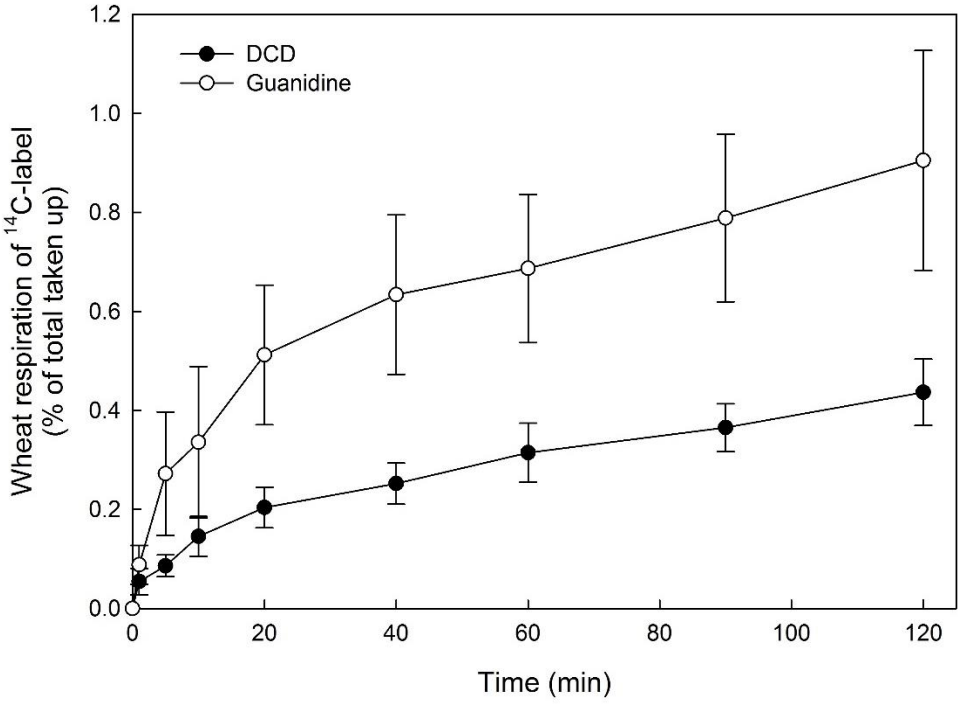

Figure 6.4 Cumulative mineralization of ^{14}C-dicyandiamide (DCD) or ^{14}C-guanidine in sterile wheat plants. Plants were exogenously supplied with each substrate (0.01 mM) for the whole 2 h monitoring period. Values represent mean ± SEM (n = 4).

6.3.3 DCD and guanidine uptake from soil rhizosphere microcosms

Wheat plants took up DCD-^{14}C and guanidine-^{14}C when grown in soil-filled microcosms (Fig. 6.5; Table 6.2), however, the rates were lower than those grown under sterile conditions. To evaluate the quantity of roots which were exposed to the injected ^{14}C labelled substrates a blue ink tracer was injected into the microcosms. From this, we estimated that 41 ± 8 and 32 ± 5% of the total root biomass was exposed to the injected ^{14}C-substrates in the Eutric Cambisol and Sapric Histosol soil, respectively.

Increasing the concentration of injected ^{14}C-DCD into soil rhizosphere microcosms increased ($p < 0.001$) rates of root uptake of the label in both soil types (Table 6.2). Although a consistently higher mean root uptake rate of DCD was observed from the Eutric Cambisol in

comparison to the Sapric Histosol at each concentration, differences were not significant (p > 0.05). After 2 h, wheat roots had acquired 0.88 ± 0.12, 0.96 ± 0.12 and 1.09 ± 0.21% of total applied DCD-^{14}C (0.01, 0.1 and 1 mM respectively) from the Eutric Cambisol and translocated 5.42 - 9.13% to the shoot material (Table 6.2); the amount acquired by wheat from the Sapric Histosol was ca. half this: 0.50 ± 0.08, 0.53 ± 0.06 and 0.44 ± 0.06% (0.01, 0.1 and 1 mM, respectively), with 7.16 - 7.80% translocated to the shoot (Table 6.2).

Increasing the concentration of injected ^{14}C-guanidine into the soil-filled microcosms increased rates of root uptake of guanidine-^{14}C in both soil types (p < 0.001; Fig. 6.5). In the Eutric Cambisol, wheat roots acquired less (p < 0.01) of the ^{14}C label added as DCD in comparison to guanidine at 0.01 mM, yet uptake rates were similar (p > 0.05) at 0.1 and 1 mM. In the Sapric Histosol, wheat roots acquired less (p < 0.05) of the ^{14}C label added as DCD in comparison to guanidine at 0.01 and 1 mM, however, uptake rates were similar at 1 mM (p > 0.05; Table 6.2). After 2 h, wheat roots had acquired 2.21 ± 0.38, 1.54 ± 0.33 and 1.00 ± 0.09% of total applied guanidine-^{14}C (0.01, 0.1 and 1 mM respectively) from the Eutric Cambisol and translocated 0.71 – 2.09% to the shoot material (Table 6.2); the total amount acquired by wheat from the Sapric Histosol was 1.26 ± 0.03, 1.05 ± 0.14 and 0.50 ± 0.08% (0.01, 0.1 and 1 mM, respectively), with 0.94 – 2.24% translocated to the shoot (Table 6.2). The percentage of acquired guanidine-^{14}C which was translocated to the shoot was lower than that of DCD-^{14}C in both soil types (p < 0.05; Table 6.2).

Unlabelled DCD was detected within the wheat shoot extracts by HPLC, confirming intact uptake and translocation of the molecule to shoot material. The concentrations of DCD recovered in shoots following injection of 1 ml of 1 mM DCD and a 2 h incubation period were 79.7 ± 19.2 and 43.7 ± 3.75 nmol DCD g^{-1} shoot DW in the Eutric Cambisol and Sapric Histosol, respectively. DCD concentrations in wheat shoots calculated from the ^{14}C data showed the same higher shoot recovery in the mineral soil and were of the same order at 25.8

± 4.71 and 9.48 ± 2.31 nmol DCD g^{-1} shoot DW in the Eutric Cambisol and Sapric Histosol, respectively. Some disparity in the absolute values may have resulted from the different groups of plants used for the two methods of measurement.

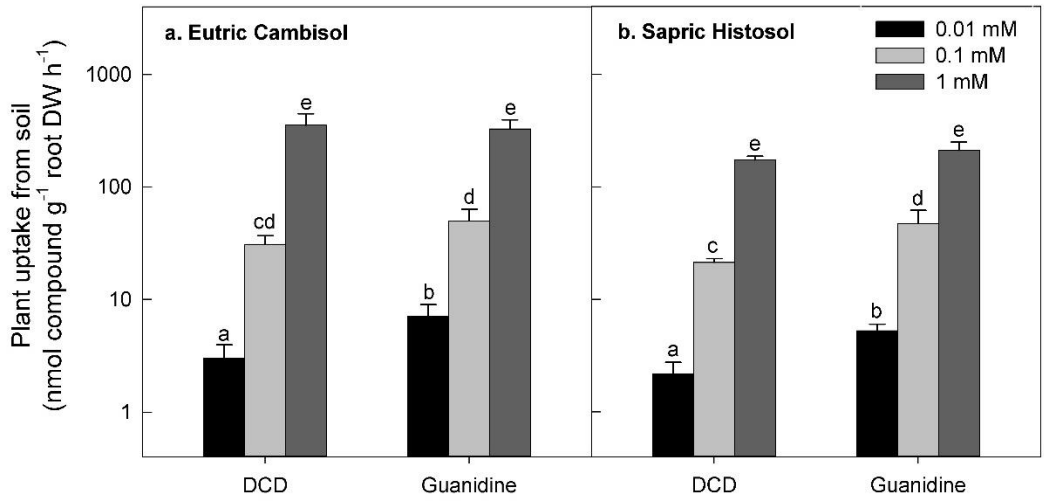

Figure 6.5 Uptake of ^{14}C-dicyandiamide (DCD) or ^{14}C-guanidine (0.01, 0.1 and 1 mM) by wheat plants grown in either a Eutric Cambisol (Panel A) or Sapric Histosol (Panel B), legend applies to both panels. Values represent means ± SEM (n = 4) and different letters indicate significant differences between means of different compounds, concentrations and between each soil type (Fisher's LSD; $p < 0.05$).

6.3.4 Microbial mineralization of DCD and guanidine within soil

Over 2 h, the amount of ^{14}C-DCD and ^{14}C-guanidine mineralized in soil was small (Fig. 6.6; Table 6.3). After 2 h in the Eutric Cambisol, less ($p < 0.01$) ^{14}C-DCD was mineralized in comparison to ^{14}C-guanidine, at 0.01 and 0.1 mM. Less ^{14}C-DCD was also mineralized at 1 mM, however, differences were not significant ($p > 0.05$). In the Sapric Histosol, more ($p < 0.01$) ^{14}C-guanidine was mineralized in comparison to ^{14}C-DCD at the lowest concentration (0.01 mM); at 0.1 and 1 mM less ($p < 0.05$) ^{14}C-guanidine was mineralized in comparison to ^{14}C-DCD. After 2 h, greater amounts of ^{14}C-DCD were degraded in the Sapric Histosol in

comparison to the Eutric Cambisol at all concentrations ($p < 0.05$); conversely, greater amounts of ^{14}C-guanidine were degraded in the Eutric Cambisol as opposed to the Sapric Histosol at all studied concentrations ($p < 0.05$).

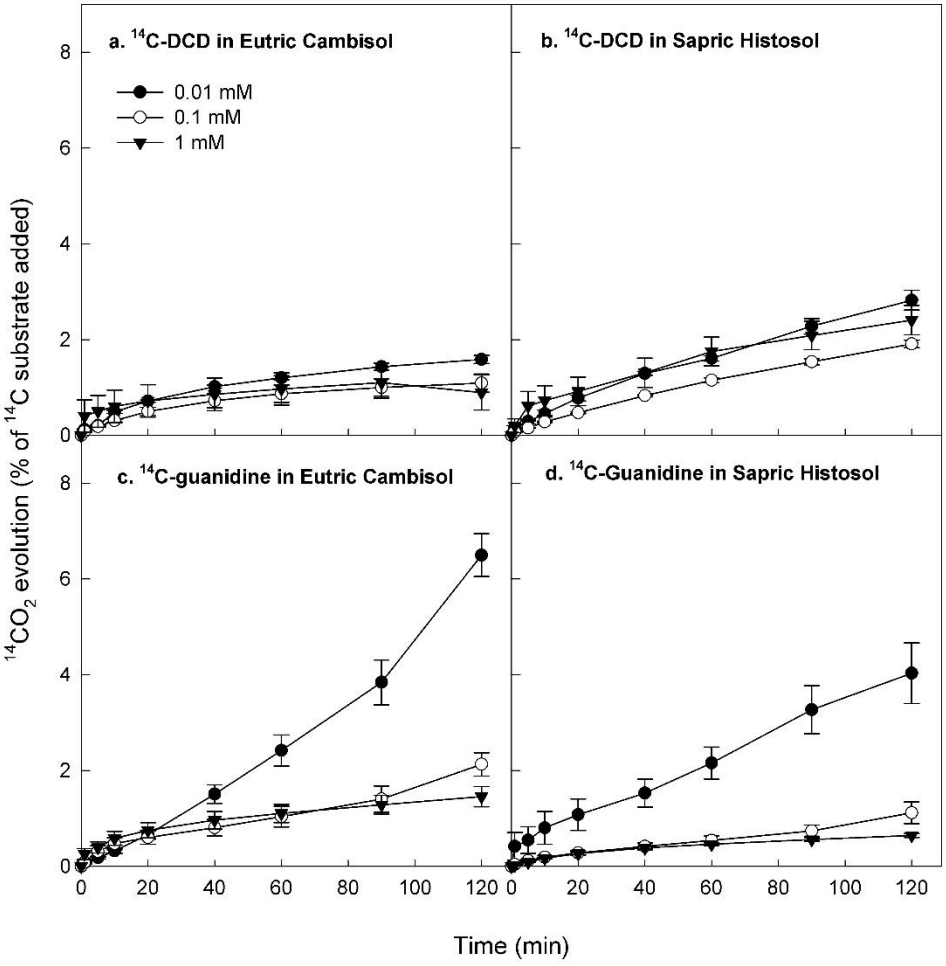

Figure 6.6 Mineralization of a) ^{14}C-dicyandiamide (DCD) in a Eutric Cambisol b) ^{14}C-DCD in Sapric Histosol c) ^{14}C-guanidine in Eutric Cambisol and d) ^{14}C-guanidine in Sapric Histosol at three concentrations (0.01, 0.1 and 1 mM). Legend applies to all panels and symbols represent means ± SEM ($n = 4$).

Table 6.3 Summary of results for microbial mineralization, microbial uptake and sorption of ^{14}C-DCD and ^{14}C-guanidine (0.01, 0.1 and 1 mM) in a Eutric Cambisol and a Sapric Histosol after 2 h. Values represent means ± SEM (n = 4).

	Soil Type	^{14}C-DCD			^{14}C-Guanidine		
		0.01 mM	**0.1 mM**	**1 mM**	**0.01 mM**	**0.1 mM**	**1 mM**
Mineralization (% of applied label)	Eutric Cambisol	1.58 ± 0.08	1.09 ± 0.08	0.89 ± 0.37	6.50 ± 0.45	2.13 ± 0.24	1.46 ± 0.21
Mineralization (% of applied label)	Sapric Histosol	2.82 ± 0.21	1.91 ± 0.08	2.40 ± 0.31	4.02 ± 0.63	1.12 ± 0.23	0.64 ± 0.04
Microbial uptake (nmol g^{-1} soil DW)	Eutric Cambisol	0.19 ± 0.004	1.68 ± 0.14	15.2 ± 0.75	0.24 ± 0.02	1.48 ± 0.09	10.8 ± 0.98
Microbial uptake (nmol g^{-1} soil DW)	Sapric Histosol	0.63 ± 0.01	5.97 ± 0.18	60.6 ± 1.64	0.68 ± 0.01	5.09 ± 0.14	50.1 ± 1.36
Sorption (nmol g^{-1} soil DW)	Eutric Cambisol	0.28 ± 0.002	3.03 ± 0.14	32.0 ± 0.57	0.28 ± 0.02	3.78 ± 0.07	41.3 ± 0.04
Sorption (nmol g^{-1} soil DW)	Sapric Histosol	0.60 ± 0.01	6.31 ± 0.16	62.2 ± 1.68	0.56 ± 0.01	7.33 ± 0.12	74.1 ± 1.06

6.3.5 Microbial uptake and sorption of DCD and guanidine within soil

The amount of ^{14}C-substrate added as DCD or guanidine present in the soil solution pool and the K_2SO_4-extractable pool is shown in Figure 6.7. As the trends in the data were similar across all concentrations, only the 0.01 mM data are presented. Increasing applied DCD or guanidine concentration from 0.01 to 1 mM increased ($p < 0.001$) the amount of DCD taken up by microbes in both soil types, at all concentrations (Table 6.3). Greater amounts of DCD and guanidine were taken up by microbes in the Sapric Histosol in comparison to the Eutric Cambisol at all studied concentrations ($p < 0.001$). In the Eutric Cambisol a greater amount of added guanidine was taken up by microbes in comparison to DCD at 0.01 mM ($p < 0.001$), and less guanidine was taken up in comparison to DCD at 0.1 and 1 mM ($p < 0.001$). In the Sapric Histosol no difference was observed in the amount of substrate taken up by microbes as DCD or guanidine at 0.01 mM ($p > 0.05$) and a lower amount of guanidine was taken up by microbes at 0.1 and 1 mM ($p < 0.01$), in comparison to DCD (Table 6.3).

Increasing either DCD or guanidine concentration from 0.01 to 1 mM increased the amount of substrate sorbed to both soil types at all studied concentrations ($p < 0.001$; Table 6.3). A greater amount of either DCD or guanidine sorbed to the Sapric Histosol in comparison to the Eutric Cambisol at all studied concentrations ($p < 0.001$). No difference was found in the amount of DCD or guanidine sorbed in either soil type at 0.01 mM ($p > 0.05$), but greater amounts of guanidine sorbed in comparison to DCD in both soil types at concentrations of 0.1 and 1 mM ($p < 0.001$; Table 6.3).

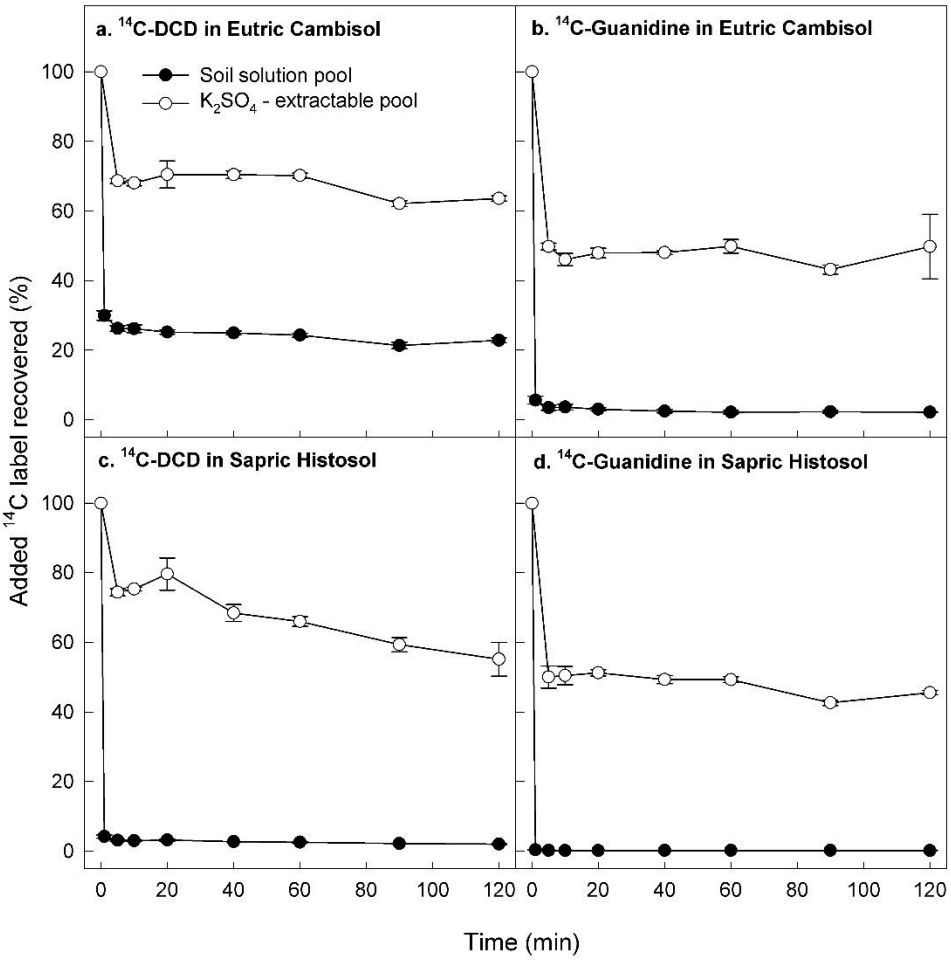

Figure 6.7 Amount of added ^{14}C label recovered in the soil solution pool and in the 0.5 M K_2SO_4-extractable pool from a) ^{14}C-dicyandiamide (DCD; 0.01 mM) added to Eutric Cambisol b) ^{14}C-DCD (0.01 mM) added to Sapric Histosol c) ^{14}C-guanidine (0.01 mM) in Eutric Cambisol and d) ^{14}C-guanidine (0.01 mM) in Sapric Histosol. Legend applies to all panels and symbols represent means ± SEM (n = 4).

6.4 Discussion

6.4.1 Plant uptake

Our results clearly demonstrate that in the absence of competing physical and biological soil processes (e.g. sorption, microbial uptake and microbial degradation), wheat roots can acquire DCD and guanidine from solution. Interestingly, rates of uptake (34.0 ± 6.29 µmol

DCD g^{-1} root DW h^{-1}; 14.3 ± 4.72 µmol guanidine g^{-1} root DW h^{-1}) at 1 mM under similar experimental conditions, were of a similar magnitude to other small N and C containing molecules found in soil (e.g. ca. 25 µmol NO_3^- g^{-1} root DW h^{-1}; ca. 23 µmol alanine g^{-1} root DW h^{-1}; Hill et al. 2013); however, rates of uptake were greater for DCD and guanidine when considering moles of N acquired (136 µmol DCD-N g^{-1} root DW h^{-1} and 42.9 µmol guanidine-N g^{-1} root DW h^{-1} compared to 25 µmol NO_3^- -N g^{-1} root DW h^{-1} and 23 µmol alanine-N g^{-1} root DW h^{-1}; Hill et al. 2013).

The mechanisms of DCD uptake and subsequent translocation within plants remain unknown. Being a synthetic compound, we hypothesise that no DCD-specific membrane transporters exist, however, it is possible that uptake could be facilitated by transporter proteins for structurally similar, naturally occurring molecules. A recent study by Eggen and Lillo (2012), found that a pharmaceutical drug used for diabetes II, metformin, had a high bioconcentration factor within seeds of *Brassica napus*. Like DCD, the drug metformin is structurally similar to the naturally occurring molecule, guanidine. The proposed mechanism of metformin entry to plant cells was via organic cation transporters (OCT), which transport naturally occurring N-containing compounds across the cell membrane. Expression of OCT has been demonstrated within several *Arabidopsis* tissues, including root tissue (Lelandais-Brière et al. 2007; Küfner and Koch 2008; Eggen and Lillo 2012). Substrates for plant OCT have not been well characterised, however, guanidine has been identified as a substrate for mammalian OCT (Cova et al. 2002). Assuming that DCD is an analogue of guanidine and acquired by the same mechanism, we would expect similar rates of uptake for both compounds. However, under sterile conditions we observed lower uptake rates of DCD compared to guanidine at 0.01 mM, similar rates at 0.1 mM and higher rates in comparison to guanidine at 1 mM. This suggests that the transporter affinity differs for the two compounds, and may also suggest there are other alternative transport pathways which need to be further investigated.

6.4.2 *Plant translocation and assimilation*

Lower amounts of DCD-^{14}C were recovered in the shoot in comparison to guanidine-^{14}C. However, the DCD-^{14}C appeared to be more evenly distributed within the shoot tissue. The low rates of mineralization of DCD- and guanidine-^{14}C within the plant over the short incubation time employed here may suggest saturation of metabolic pathways and thus that plant capacity to use the C and N acquired in these molecules is low. Recovery of intact DCD in wheat shoots by HPLC perhaps supports this view. However, preferential accumulation of structurally similar metformin, in seeds of *Brassica napus* and the occurrence of other guanidine derivatives, including arginine, in seeds may suggest storage of DCD and guanidine i.e. a lack of metabolic pathways which are directly connected to respiration (Ngamga et al. 2007; Eggen and Lillo 2012).

6.4.3 Competition for DCD and guanidine in the rhizosphere

Wheat was able to acquire ca. 0.5-1.0% of the ^{14}C applied as DCD and ca. 0.5-2% of that applied as guanidine from soil within 2 h when in competition with the rhizosphere microbial community and sorption processes. Although realistic DCD soil solution concentrations were chosen for this study, direct injection into the rhizosphere may have resulted in a greater amount of root surface area exposed to NI-containing soil solution than may be expected under field conditions. Actual DCD concentrations in the rhizosphere may vary according to NI application method, weather conditions, crop type, soil temperature and moisture and time since application. As only the C within molecules was isotopically labelled and detected within plant tissue, the results of the ^{14}C-DCD uptake study do not unequivocally demonstrate that DCD was taken up intact from soil without prior lysis by soil microbes, or whether it remained intact once inside the plant without further degradation or transformation

of the DCD molecule. Detection of unlabelled DCD within wheat shoots via HPLC, however, shows that intact uptake of DCD by plants took place.

Our results suggest that soil type is a regulator of DCD and guanidine bioavailability, with wheat acquiring consistent numerically (although not statistically) greater amounts of DCD-^{14}C and guanidine-^{14}C from the mineral Eutric Cambisol in comparison to the organic Sapric Histosol. A combination of a greater microbial uptake and sorption in the Sapric Histosol similarly suggests a lower availability for plant acquisition when compared to the Eutric Cambisol. The Sapric Histosol has a greater cation exchange capacity and more soil organic matter (which has been identified as important source of DCD binding domains (Jacinthe and Pichtel, 1992; Zhang et al. 2004)) in comparison to the Eutric Cambisol, which may have led to greater sorption in this soil. The Sapric Histosol also had a greater microbial biomass compared to the Eutric Cambisol on a soil weight basis, which may have caused greater amounts of DCD and guanidine to be taken up by microbes in this soil.

In comparison to some other simple C substrates (e.g. amino acids, sugars) and the level of sorption to soil particles, the mineralization of DCD and guanidine by soil microbes was very slow (Hill et al. 2008; Wilkinson et al. 2014). This might be expected considering that neither soil has previously been exposed to the synthetic nitrification inhibitor, DCD. The dissimilar pattern of DCD and guanidine mineralization, particularly at low concentrations in the Eutric Cambisol supports the tenet that DCD is not rapidly extracellularly degraded to guanidine. The greater degradation of DCD in the Sapric Histosol in comparison to the Eutric Cambisol may be explained by the greater microbial biomass on a soil weight for weight basis, although reasons for the lower rates of guanidine mineralization in the organic soil compared to the mineral soil are unclear. The low rates of guanidine uptake and mineralization at higher guanidine application rates ($\geq$ 0.1 mM) may also suggest that although naturally found in the microbial community, the capacity to internally assimilate high amounts of guanidine limits its

metabolic conversion to CO_2. This supports the conclusions of Rajbanshi et al. (1992) in that the microbial community requires longer time periods than 2 h to adapt to utilising DCD in soil. However, we cannot exclude the possibility that both DCD and guanidine are metabolised largely by pathways which do not feed into respiration.

Our results support the suggestion of Kelliher et al. (2014) that plants may play a role in reducing the half-life of DCD within soils, and this may be more pronounced in mineral soils as opposed to organic soils where plant uptake rates were greater due to lower amounts being sorbed and taken up by microbes. Interestingly, they also suggest that plants are able to derive some additional N from soils where DCD has been applied, and that guanidine (and perhaps other similar N-rich molecules) can be added to the growing list of naturally occurring N forms which plants are able to acquire from soil and metabolise. However, we hesitate to suggest that this has a significant role in plant N nutrition.

6.4.4 DCD entry into the food chain

There are three obvious routes of entry for DCD into meat or milk products: by i) consumption of pasture which has intercepted DCD on the foliage during spray application of the NI ii) direct livestock consumption of pasture or forage (which has acquired DCD from the soil as demonstrated here) and iii) ingestion of soil particles containing DCD.

Based on a dairy cow consuming 15 kg DM day^{-1} of grass (McDonald et al. 1996), with a standing biomass of 2000 kg DM ha^{-1} (O'Donovan and Dillon 1999), following a spray application of DCD (10 kg ha^{-1}), with 5% of the total applied DCD intercepted via the canopy (Kim et al. 2012) we estimate that 3.5 g of DCD cow^{-1} day^{-1} could be consumed if allowed to graze immediately following application. Assuming that wheat is representative of other grasses and extrapolation of uptake and respiration rates to 24 h (with 50% translocation to shoots over this period), we estimate that 0.43 mg DCD cow^{-1} day^{-1} and 0.15 mg DCD cow^{-1}

day^{-1} could be ingested following consumption of grass grown on the Eutric Cambisol and the Sapric Histosol, respectively. To estimate the amount of DCD consumed via soil ingestion we assume an even distribution of DCD in the top 10 cm of soil (soil bulk density of 1.10 and 0.31 g cm^{-3} for the Eutric Cambisol and Sapric Histosol, respectively) with removal due to plant uptake as above, and microbial mineralization at 1.30% and 5.93% (Eutric Cambisol and Sapric Histosol, respectively) of applied DCD over 24 h (measured over this period by Scowen, M; unpublished). Based on 2% of the cow's DM intake being soil (Thornton and Abrahams, 1983), 2.68 and 9.1 mg DCD cow^{-1} day^{-1} could be ingested with soil in the Eutric Cambisol and Sapric Histosol, respectively. Our calculations are based on a number of assumptions and upscaling laboratory data to field conditions, therefore, care should be taken when considering these estimations. Further research is required to elucidate how application methods (e.g. liquid vs. granular formulations) and environmental conditions (e.g. movement of NI into the root zone due to irrigation or rainfall) may influence pasture plant acquisition of this NI under field conditions.

Based on our estimates the magnitude of risk for DCD entry into the food chain via cattle follows the trend: pasture interception of DCD > soil ingestion of DCD > plant acquisition of DCD. However, the greatest risk pathway (canopy interception) would be transient (e.g. under high rainfall) and easily controlled by preventing grazing immediately following DCD application to pasture. Controlling the amount of DCD ingested beyond this point represents more of a challenge for out-grazing livestock and plant uptake may be a more significant pathway over longer periods. The potential for DCD to enter raw foods destined for direct human consumption (e.g. salad vegetables) also requires further investigation as the concentrations of DCD may be higher than in dairy products. Further, if like some other guanidine derivatives DCD accumulates in seeds, application of DCD to arable grain, oilseed

or pulse crops could result in further direct DCD entry into the food chain due to the capacity of plants to acquire DCD from soil through roots (Ngamga et al. 2007; Eggen and Lillo, 2012).

6.5 Acknowledgments

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Chapter 7

Effect of time-since DMPP application on mitigating N_2O emissions from sheep urine patches

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KAM, DLJ and DRC designed and conceived the experiment, KAM conducted the experimental work, analysed the results and prepared the manuscript.

Abstract

Nitrification inhibitors are a potential technology to mitigate N_2O emissions from the urine patches of grazing animals. At present there is limited information regarding the efficacy of the nitrification inhibitor 3,4-dimethylpyrazole phosphate (DMPP) in reducing N_2O emissions from ruminant urine patches, as opposed to the well-studied nitrification inhibitor, dicyandiamide. In practical terms, urine patches would be deposited to soil at various times following the application of a nitrification inhibitor to soil. We hypothesised that the effectiveness of DMPP in reducing cumulative N_2O emissions would decrease the longer the time since DMPP application. This study utilised an automated closed chamber technique, to monitor fluxes of N_2O from sheep urine patches (725 kg N ha^{-1}; 150 ml; 300 cm^2) deposited to a Eutric Cambisol, where DMPP was applied (1 kg ha^{-1}) on the same day, 2 weeks before and 4 weeks before urine application. Fluxes were monitored continuously from 4 weeks before to 9 weeks after urine application. DMPP was found to be ineffective in reducing cumulative N_2O emissions when applied prior to, or even when applied at the same time as the sheep urine. Some effect of DMPP in delaying the accumulation of NO_3^- was observed, with effects being greater the shorter the time since DMPP application. The temporal dynamics of N_2O fluxes were also altered where DMPP was applied on the same day as the urine. The N_2O emission factors ranged from ca. 0.33-2.49% of the applied N, with an average of 0.81 ± 0.22% across all chambers. This was lower than the default IPCC emission factor of 1% for sheep, despite the fairly high N application. Heterogeneity in soil conditions were deemed responsible for the large spatial variability of N_2O emissions observed in this study. Further work is required to determine whether DMPP can reduce emissions from urine patches at a range of N concentrations, under different climatic conditions and with a range of nitrification inhibitor application methods.

Key words: diurnal; grassland; greenhouse gases; nitrogen cycle; sustainable intensification.

7.1 Introduction

The agricultural sector is the second largest source of greenhouse gases in the UK (following the energy sector; Webb et al., 2014), contributing ca. 8% of the UK's total (direct and indirect) anthropogenic emissions (Gill et al., 2010). The agricultural sector will need to contribute to the ambitious GHG emissions reduction target set by the UK Climate Change Act, i.e. to reduce emissions by 80% from base year levels, by 2050. The sustainable management of livestock excreta will form an important component of reducing agricultural emissions, especially under projected increases in global meat consumption (Davidson, 2009). Pastures cover a greater portion of the earth's surface compared to arable soils (7% and 3%, respectively; Oenema et al., 2005) and urine patches deposited to pastures are known hotspots for N cycling, where microbial transformations (e.g. nitrification and denitrification) can result in elevated N_2O emissions (de Klein and van Logtestijn, 1994; Carter, 2007). Excreta (dung and urine) deposited to pasture soils account for ca. 40% of the global N_2O emissions arising from animal production systems (Oenema et al., 2005).

The use of nitrification inhibitors within grazed pastures offers a potential means of reducing N losses whilst promoting nitrogen use efficiency (Di and Cameron, 2002; Di and Cameron, 2007). By delaying the first and rate-limiting step of nitrification (the oxidation of NH_4^+ to NO_2^-; Chaves et al., 2006; Fiencke and Bocke, 2006; Benckiser et al., 2013), nitrification inhibitors enable nitrogen to persist in the soil in the ammoniacal form for longer. This increases opportunity for plant acquisition of applied N, reduces NO_3^- leaching and can reduce N_2O emissions from both nitrification and denitrification processes. Two of the most widely researched nitrification inhibitors include dicyandiamide (DCD) and 3,4-dimethylpyrazole phosphate (DMPP; Liu et al., 2013), where the latter is a newer product, developed by BASF (Ludwigshafen, Germany; Barth et al., 2001; Zerulla et al., 2001). In addition, reviews on the efficacy of nitrification inhibitors have revealed a greater effectiveness

in reducing N_2O emissions from grasslands, in comparison to other land use types (Akiyama et al., 2010; Gilsanz et al., 2016).

There have been a greater number of studies exploring the effect of DCD in reducing N_2O emissions from urine patches compared to DMPP (e.g. Di and Cameron, 2003; deKlein et al., 2011; Misselbrook et al., 2014; Bell et al., 2015). The use of an alternative nitrification inhibitor to DCD within pasture soils may be necessary, due to evidence of plant acquisition of DCD, which may lead to contamination of milk products from dairy pasture soils (Marsden et al., 2015). DMPP may be a suitable alternative to DCD for use in pasture soils as it has a lower phytotoxicity and slower degradation rates in soil (Weiske et al., 2001; Zerulla et al., 2001).

DMPP has been shown to be effective in reducing N_2O emissions from fertilisers (Linzmeier et al., 2001; Menéndez et al., 2012; Bell et al., 2015) and livestock slurry (Chadwick et al., 2001; Hatch et al., 2005; Merino et al., 2005) but there are few studies investigating DMPP efficacy where ruminant urine is the nitrogen source (e.g. Di and Cameron, 2011; Di and Cameron, 2012; Gilsanz et al., 2016). When applied at the same time as a cattle urine application (1000 kg N ha^{-1}), a liquid DMPP (5 kg ha^{-1}) application reduced N_2O emissions by 66% (Di and Cameron, 2012). Misselbrook et al. (2014) and Barneze et al. (2015) investigated the N_2O reduction efficacy of pyrazole derivatives (which are structurally similar to DMPP) applied to cattle urine, however, no significant reduction in N_2O emissions were observed. DMPP performance has been found to be best under cold and wet conditions (Menéndez et al., 2012), therefore, whether DMPP is effective under warmer soil conditions is still unclear.

This study was carried out to determine whether DMPP is effective in reducing N_2O emissions arising from pasture soils influenced by sheep urine, under UK summer conditions. Given that sheep will deposit urine at various times following an application of DMPP, and that cost and labour associated with application will increase with the need for repeated

applications, we wanted to assess the duration of DMPP efficacy in reducing N_2O emissions. We used a high frequency automated GHG monitoring system to evaluate the efficacy of a liquid DMPP application in reducing N_2O emissions from sheep urine applied immediately after, 2 weeks after and 4 weeks after DMPP application. We hypothesised that N_2O reduction efficacy would reduce with the greater the time-since application, due to degradation and immobilization of the nitrification inhibitor within the soil.

7.2 Materials and Methods

7.2.1 Field site

The study site was established on a lowland grazed grassland at Henfaes Research station, Abergwyngregyn, North Wales (53°14′N, 4°01′W). The soil at the site is classified as a Eutric Cambisol, and is of glacial till (deposited ca. 10 000 years ago) in origin. The recent history includes moderate fertilizer applications, light grazing by Welsh Mountain ewes and reseeding in 1990 with a *Lolium perenne* L. and *Trifolium repens* L. mix. To prevent the effects of recent livestock excretal depositions on monitored gas fluxes, livestock were excluded from the study site five months prior to treatment application.

7.2.2 Soil sampling and analysis

To provide the background soil characteristics (Table 7.1) of the study site, soil was sampled (0-10 cm, 1.5 cm diameter) in triplicate, at the block level (n = 3). The bulk density was measured by inserting a 100 cm^3 metal ring into the ground, oven-drying the removed soil core (105°C; 24 h), and sieving (< 2 mm) the dry soil to remove and weigh stones. Gravimetric moisture content was determined by oven drying soil (105°C; 24 h) and organic matter was determined by loss-on-ignition (450°C; 16 h; Ball, 1964). Soil pH and electrical conductivity (EC) were measured with standard electrodes, submerged in 1:2.5 (w/v) soil-to-distilled water

suspensions. The total soil C and N content of oven-dried and ground samples were determined using a TruSpec® Analyzer (Leco Corp., St. Joseph, MI); pasture foliar C and N contents, before and during the study, were also analysed using the same instrument. Dissolved (1:5; w/v; soil-to-0.5M K_2SO_4) total C and N concentrations in soil were determined by the method of Jones and Willet (2006), using a multi N/C 2100S analyser (AnalytikJena, Jena, Germany). The $CHCl_3$-fumigation-extraction method of Voroney et al. (2008), was used to determine microbial biomass C and N, where the resulting C and N in the extracts were analysed as described previously, using K_{EC} and K_{EN} correction factors of 0.35 and 0.5, respectively. Concentrations of P, NO_3^- and NH_4^+ were determined in 0.5 M K_2SO_4 soil (0-5 cm) extracts using colorimetric methods described in Murphy and Riley (1962), Miranda et al. (2001) and Mulvaney (1996), respectively. Exchangeable cations (Na, K, Ca) were measured using a Sherwood Model 410 flame photometer (Sherwood Scientific Ltd., Cambridge, UK) in 1 M NH_4Cl extracts (1:5, w/v, soil-to-1 M NH_4Cl).

7.2.3 Sheep urine collection and analysis

Welsh Mountain ewes (n = 6) were housed in individual pens, containing slatted plastic flooring specifically designed for sheep (Rimco Ltd., Yorkshire, UK). The pen flooring was slightly raised above the ground, allowing room for urine collection trays. The sheep were allowed free access to water and provided with a diet (*ad libitum*) of freshly cut pasture similar in composition to the field site. During the urine collection period individual urine volumes were recorded, the samples were filtered (0.45 µm) and frozen (unacidified). The urine collected from all sheep were bulked (to provide sufficient volume of homogenous composition) and thoroughly mixed before application to the soil. Total dissolved organic C, total N, P, NO_3^-, NH_4^+ and cations in the sheep urine were analysed as described for the soil extracts, and the urea content was determined via the method of Orsonneau et al. (1992).

Table 7.1 Properties of the Eutric Cambisol at the field site receiving urine and DMPP applications. Values represent means ± SEM (n = 3) and results are reported on a dry weight basis.

Eutric Cambisol properties (0-10cm)	
Texture	Sandy Clay Loam
Bulk density (g cm^{-3})	1.08 ± 0.05
Gravimetric moisture content (%)	21.5 ± 0.9
Organic matter (%)	9.99 ± 0.32
pH	5.91 ± 0.03
EC (µS cm^{-1})	32.7 ± 0.9
Total C (%)	3.99 ± 0.01
Total N (%)	0.34 ± 0.01
C:N ratio	11.6 ± 0.2
Dissolved organic C (mg C kg^{-1})	170 ± 25
Total dissolved N (mg N kg^{-1})	23.1 ± 2.8
Microbial biomass C (g C kg^{-1})	2.44 ± 0.30
Microbial biomass N (mg N kg^{-1})	171 ± 10
Extractable NO_3^- (mg N kg^{-1})	3.97 ± 0.45
Extractable NH_4^+ (mg N kg^{-1})	3.23 ± 0.99
Extractable P (mg P kg^{-1})	3.37 ± 0.44
Exchangeable Na (mg kg^{-1})	43.8 ± 1.1
Exchangeable K (mg kg^{-1})	140 ± 12
Exchangeable Ca (g kg^{-1})	1.07 ± 0.09

7.2.4 Experimental design, treatments and application dates

The study was laid out in a randomised block design, consisting of five treatments (n = 3) as follows: 1) no urine application (control), 2) sheep urine only, 3) sheep urine plus DMPP applied at the same time, 4) sheep urine plus DMPP applied 2 weeks before urine application, and 5) sheep urine + DMPP applied 4 weeks before urine application, hereafter referred to as NU, SU, SU + DMPP, SU + DMPP 2, and SU + DMPP 4, respectively. For each treatment, urine patches were duplicated next to each flux chamber, allowing one patch for monitoring

greenhouse gases from undisturbed soils and another for soil sampling. Sheep urine (150 ml) was poured onto respective plots using a fixed template as an area guide (300 cm^2), to represent an average sheep urine volume and patch size (Doak, 1952). Liquid applications of DMPP (250 ml; 0.1 g DMPP l^{-1}) were sprayed (a normal nitrification inhibitor application method for grazed grasslands; Di and Cameron, 2011) by hand onto respective plots (2500 cm^2 across the chamber basal area), at a frequently used equivalent rate of 1 kg DMPP ha^{-1} (Zerulla et al., 2001). The first DMPP treatment application to respective plots (4 weeks before urine application; SU + DMPP 4) took place on 29/6/2015, the second DMPP treatment application (2 weeks before urine application; SU + DMPP 2) was applied on 13/7/2015, and the final DMPP treatment application (SU + DMPP) took place on 27/7/2015. Following this, sheep urine was applied to all plots except for the control on the same date as the final DMPP application.

7.2.5 Greenhouse gas emission monitoring

An automated high frequency (8 flux measurements per day/night cycle) greenhouse gas monitoring system (Queensland University of Technology, Institute for Future Environments, Brisbane, Australia), as described in Scheer et al. (2014), was used to measure fluxes of N_2O from the urine treated soils. As the automated system consisted of 12 chambers only (i.e. enough for 4 treatments, where n = 3), fluxes of N_2O from the NU treatment were measured manually using the conventional static chamber technique (1 flux measurement as close to daily as possible); fluxes of N_2O were expected to be lower and less variable from the NU treatment, compared to urine treatments, as no N was applied. We, therefore, expected the difference in measurement techniques to cause minimal bias in the results.

For the automated chambers, stainless steel chamber bases (2500 cm^2 basal area) were inserted into the soil (10 cm depth), and chambers (50 cm × 50 cm × 15 cm) were clamped to

the bases. The chambers opened and closed via pneumatic actuators, where one block of chambers would close sequentially every 1 h. While one block of chambers were closed, the other two blocks of chambers were open, allowing ambient conditions to be restored within the chambers. Chamber headspace gas samples were pumped (ca. 200 ml min^{-1}) to a sampling unit, through Teflon tubing. The sampling unit housed a LI-COR LI-820 non-dispersive infrared gas analyser (LI-COR, St Joseph, MI, USA) to measure CO_2. Samples were then passed through an Ascarite (sodium hydroxide coated silica) filter before being pumped to a gas chromatograph (SRI 8610C, Torrance, USA), equipped with a ^{63}Ni electron capture detector (ECD) and flame ionization detector (FID) to measure N_2O and CH_4 concentrations, respectively. Over the 1 h chamber closure period, each chamber (n = 4) was sampled at 3 minute intervals followed by a calibration standard (500 ppb N_2O; 880 ppm CO_2; 3 ppm CH_4; ± 2 % of the certified value, BOC gases, Liverpool, UK). For each chamber, this results in headspace concentration measurements once every 15 min, over the course of 1 h. A full cycle takes 3 hours to complete, resulting in (where uninterrupted measurement occurs) 8 flux measurements per 24 h.

The manual gas samples from the NU treatment were taken by placing polypropylene upturned buckets (ca. 26 cm in height) onto collars (26 cm diameter), inserted to a depth of 10 cm. The chambers were fitted with a re-sealable vent to allow pressure equalisation when placing chambers onto bases, and were fitted with Suba-Seals ® (Sigma, Gillingham, UK) to allow headspace gas sampling. Headspace samples were taken with a syringe every 15 min over the period of 1 h, to match the automated system. Samples were stored in pre evacuated 20 ml glass vials, before being analysed on a Perkin Elmer 580 Gas Chromatograph served by a TurboMatrix 110 auto sampler (Perkin Elmer, CT, USA). The system contained two Elite-Q PLOT megabore capillary columns, with split injection. One column was linked to an ECD (375ºC), and the other to an FID (350ºC) with methaniser, and the oven temperature was 50ºC.

Fluxes (from both methods) were measured for a total of 13 weeks, (beginning 4 weeks before urine application and ending 9 weeks after urine application). Prior to the start of the experiment, standard gases were analysed by both GCs (laboratory and field system) and shown to be not significantly different (data not shown).

7.2.6 Ancillary measurements

To support the emission measurements, several ancillary measurements were made. Soil cores (0-5 cm) were taken from replicated plots on several dates throughout the experiment. Gravimetric moisture content and 1:5 (w/v) soil-to-0.5 M K_2SO_4 extractions were conducted on the resulting soil samples. The extracts were analysed for NO_3^-, NH_4^+, extractable dissolved organic C and total N, as described previously. In order to allow sampling of the soil solution from within the chambers in a non-destructive manner, Rhizon suction samplers (2.5 cm diameter, 5 cm porous part, 12 cm length tubing; Rhizosphere Research Products, Wageningen, Netherlands) were inserted into the centre of the chamber plots. Soil solution samples were collected periodically from the Rhizons through a needle inserted into an evacuated 9 ml plastic container, when soil conditions were moist enough for successful sample collection. The soil solution samples were also analysed for NO_3^-, NH_4^+, dissolved organic C and dissolved total N, as described previously. Soil moisture sensors (Acclima SDI-12 digital TDT® sensors; Acclima Inc., ID, USA) were inserted diagonally through the urine patch (but were in situ several weeks prior to urine application) within the chambers, in order to measure soil moisture non-destructively. The pasture foliar N content was measured within the chambers at block level prior to urine application, and pasture biomass and foliar N content was measured in each chamber at 3, 6 and 9 weeks following urine application. The grass was also cut in the duplicated plots at the same time as the chambers to ensure consistency. Air temperature was monitored inside and outside the chambers using Thermochron iButtons®

(iButtonLink, LLC, WI, USA) logging temperature every 1 h. Rainfall, soil (0-10 cm) and air temperature were monitored hourly at a weather station near to the field site.

7.2.7 Data processing and statistical analysis

Fluxes of N_2O were calculated from each chamber as described in Scheer et al. (2014). The combination of three or four data points on the slope of N_2O increase or decrease, over the 1 h chamber closure period, which provided the best R^2 value was used in the final reported fluxes. Cumulative N_2O emissions were calculated by trapezoidal integration (from the point of urine application onwards) and emission factors were calculated using the following equation:

$$EF = (\text{treatment } N_2O\text{-}N - \text{control } N_2O\text{-}N) / \text{Total N applied} \times 100\% \qquad \text{(Eqn. 1)}$$

Differences in cumulative N_2O emissions were analysed by Welch's test followed by Games-Howell pairwise comparisons, due to the data not conforming to homogeneity of variance assumptions required for a parametric statistical procedure, even after data transformation. After assessing the normality (Shapiro Wilk test) and homogeneity of variance (Levene's test) assumptions, the N_2O emission factors were compared via one way ANOVA, with Tukey's post-hoc test, following log transformation of the data. The same analysis was conducted for extractable NH_4^+, NO_3^-, N and dissolved organic C at each sampling point. The soil solution NH_4^+, NO_3^-, N and dissolved organic C had a variable *n*, ranging from 1-3, due to successful sample collection being dependent on soil conditions being moist enough. Therefore, numerical trends in the soil solution NH_4^+, NO_3^-, total N and dissolved organic C data were reported rather than statistical differences. The soil solution mineral N data allowed determination of whether N processing was occurring at similar rates in the chambers and the

duplicated plots used for the soil coring, as the Rhizon samplers were located directly inside the chambers. Pasture biomass and foliar N content were also analysed by ANOVA, as above, on each of the three biomass cutting dates. All statistical analyses were conducted in Minitab 17.0 (Minitab Inc., State College, PA, USA).

7.3 Results

7.3.1 Urine characteristics

The composite sheep urine sample contained 14.5 g N l^{-1} and 28.7 g C l^{-1}, which resulted in an equivalent N application rate of 725 kg N ha^{-1} in all applied urine patches. The urine contained 12.4 g urea-N l^{-1}, 0.4 mg NO_3^--N l^{-1}, 211 mg NH_4^+-N l^{-1}, 5.5 mg P l^{-1}, 1.85 g Na l^{-1}, 13.9 g K l^{-1} and 284 mg Ca l^{-1}. The urine had a pH and EC of 8.48 and 25.3 mS cm^{-1}, respectively. Out of a total of 40 urine events between the 6 sheep, the mean and range of individual urine volumes were 104 (18-397) ml per urination event.

7.3.2 Weather data and soil moisture

The hourly rainfall, soil temperature (0-10 cm) and water-filled pore space data throughout the study period can be seen in Fig. 7.1. The first DMPP application on the 29/06/15 for treatment SU + DMPP 4, was applied under warm and dry conditions. The second DMPP application on the 13/07/15, for treatment SU + DMPP 2, was applied during a period of rainfall. The final DMPP and urine applications (27/07/15) were also under periods of rainfall. The total rainfall over the course of the study was 137 mm, and the average soil temperature (0-10 cm) was 16 ºC, ranging from 11-20 ºC. The average WFPS over the course of the study 0was 27%, and ranged from 5-69%, with minimum values at the beginning of the experiment following an extended dry period, and maximum values occurring after rainfall events.

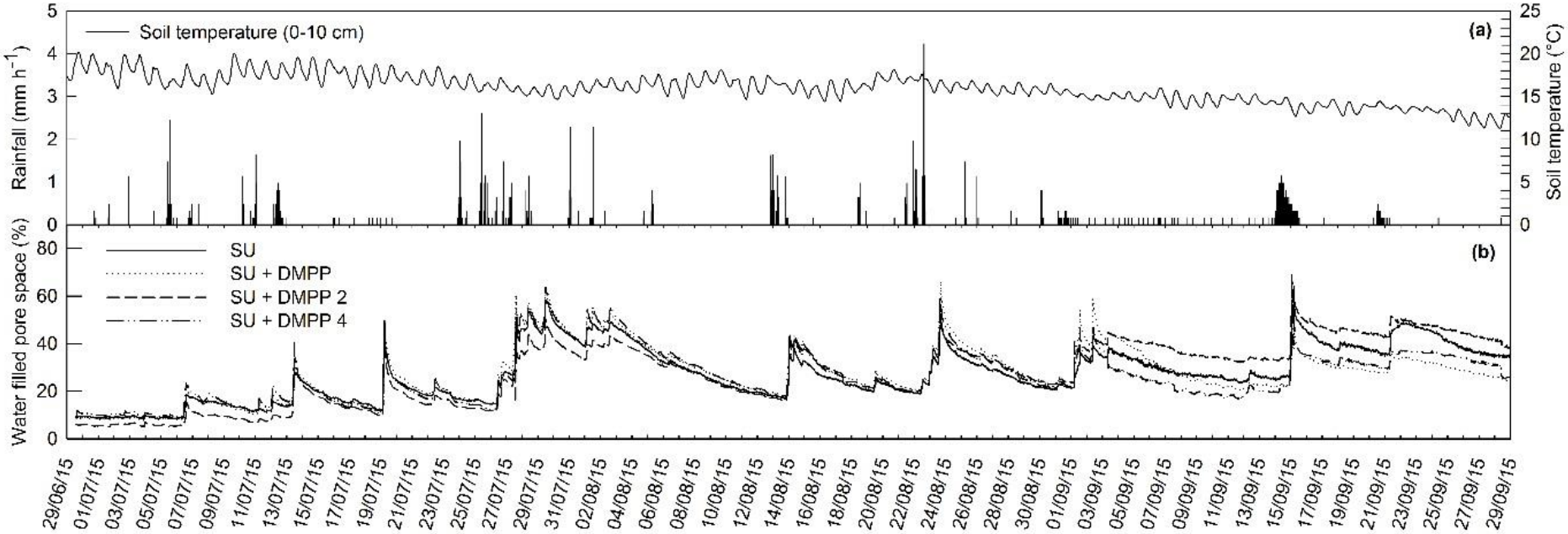

Figure 7.1. Rainfall, soil temperature and soil water-filled pore space during the course of the field trial. Hourly rainfall and soil (0-10 cm) temperature are shown in panel a) and water-filled pore space within the automated chambers are displayed in panel b). The date of measurements are displayed in dd/mm/yy format and legends apply to each respective panel only.

7.3.2 Nitrous oxide emissions

The fluxes of N_2O from all treatments can be seen in Fig. 7.2. When considering the whole automated chamber data set (8420 flux measurements), 79% of the headspace concentration data had an $R^2 > 0.95$, 88% had an $R^2 > 0.90$ and 97% of the data had an $R^2 > 0.80$, indicating the increase/decrease in N_2O within the chamber headspace over time fitted well to the assumption of linearity. In the vast majority of cases three, rather than four, data points of increase in chamber headspace concentration over time resulted in a higher R^2. As can be seen in Fig. 7.2a, the main N_2O peak from the SU treatment occurred within the first nine days following urine application, with a maxima of 264 ± 179 μg N_2O-N m^{-2} h^{-1}. DMPP was effective in reducing this initial N_2O peak when applied at the same time as the urine (Fig. 7.2b), however, a broader, shallower peak began around the 30/08/15, which was not evident in the SU treatment. In both treatments, SU + DMPP 2 and SU + DMPP 4, this initial peak was similar in size (Fig. 7.2c and d, respectively) with maximum peaks in the first nine days of 192 ± 151 and 231 ± 89 μg N_2O-N m^{-2} h^{-1}, respectively. Notably the variation in N_2O emissions is higher in Fig. 7.2c and 2d compared to 7.2a and 7.2b, and a strong diurnal trend in N_2O fluxes was observed.

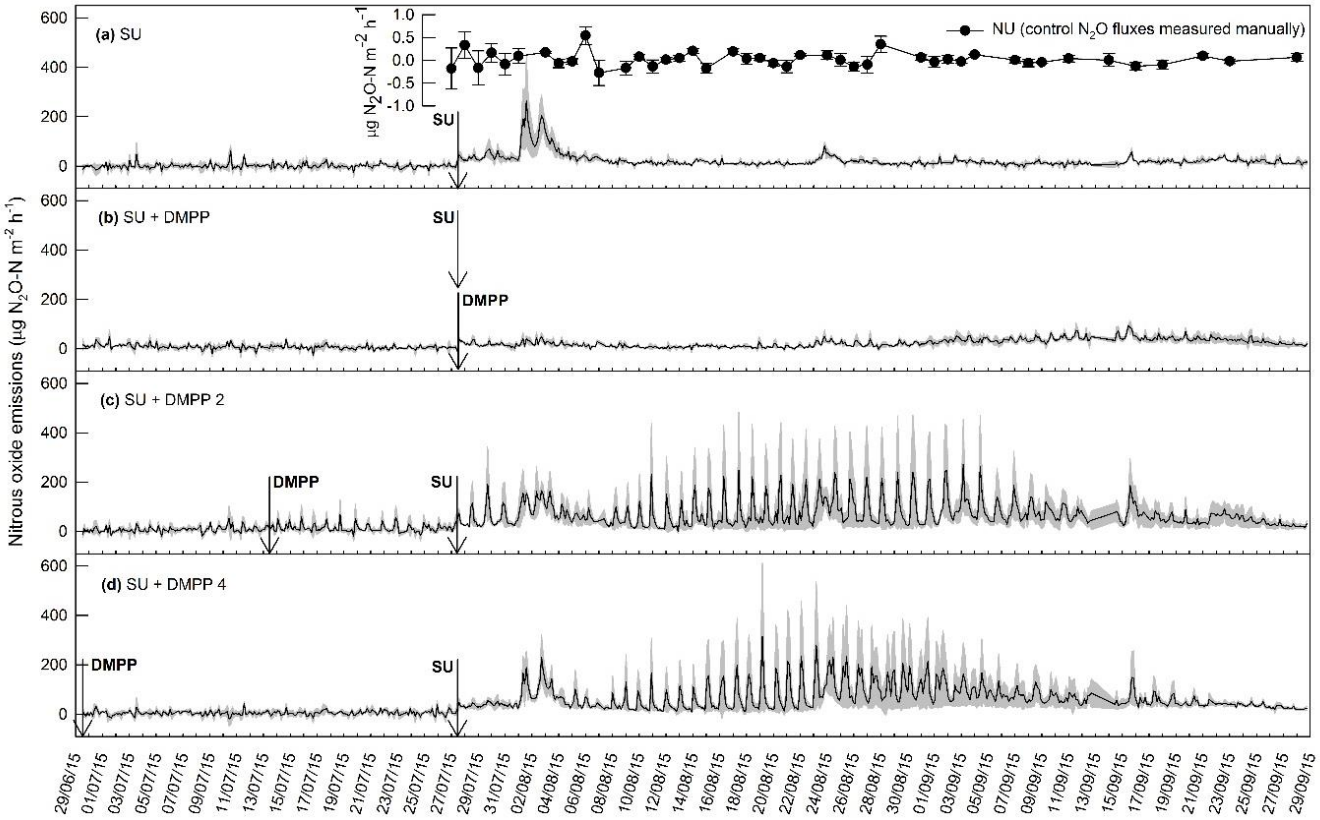

Figure 7.2. N_2O fluxes following sheep urine and DMPP applications to a Eutric Cambisol, where panel a) contains fluxes from treatments SU and NU and panels b), c) and d) display fluxes from SU + DMPP, SU + DMPP 2 and SU + DMPP 4, respectively. In each panel the black line represents the mean flux (n = 3) and the grey shaded area represents the upper and lower bounds of the SEM. In treatment NU, error bars denote SEM. Arrows indicate the timing of either sheep urine (SU) or DMPP applications and the dates along the x-axis are in dd/mm/yy format.

7.3.3 Cumulative N_2O emissions and emission factors

Cumulative N_2O emissions were calculated from the point of urine application onwards, and can be seen in Fig. 7.3. Mean cumulative emissions ranged from 0.4-1.0 kg N_2O-N ha^{-1}. Due to the high variability of the SU + DMPP 2 and SU + DMPP 4, homogeneity of variance was not achieved across the treatments, therefore, Welch's test was used to assess differences. The results of the Welch's test were positive ($p < 0.05$) both with and without the removed 'high emitting' replicates; the statistical groupings from the Games-Howell test can be seen in Fig. 7.4. Here it can be seen that the SU and SU + DMPP treatments emitted significantly more N_2O in comparison to NU. Due to the large variability in the SU + DMPP 2 and SU + DMPP 4 treatments (and the low value for *n* when one replicate is removed), no differences were observed between these treatments and NU. In contrast to our hypotheses, DMPP did not reduce the cumulative N_2O emissions from sheep urine patches when applied at the same time as the urine, or when applied 2 and 4 weeks before urine application. The N_2O emission factors were 0.41 ± 0.05, 0.43 ± 0.04, 1.22 ± 0.56 and 1.19 ± 0.65% of the applied N for SU, SU + DMPP, SU + DMPP 2 and SU + DMPP 4 treatments, respectively (without removal of outliers). No significant differences ($p > 0.05$) were found between the treatments.

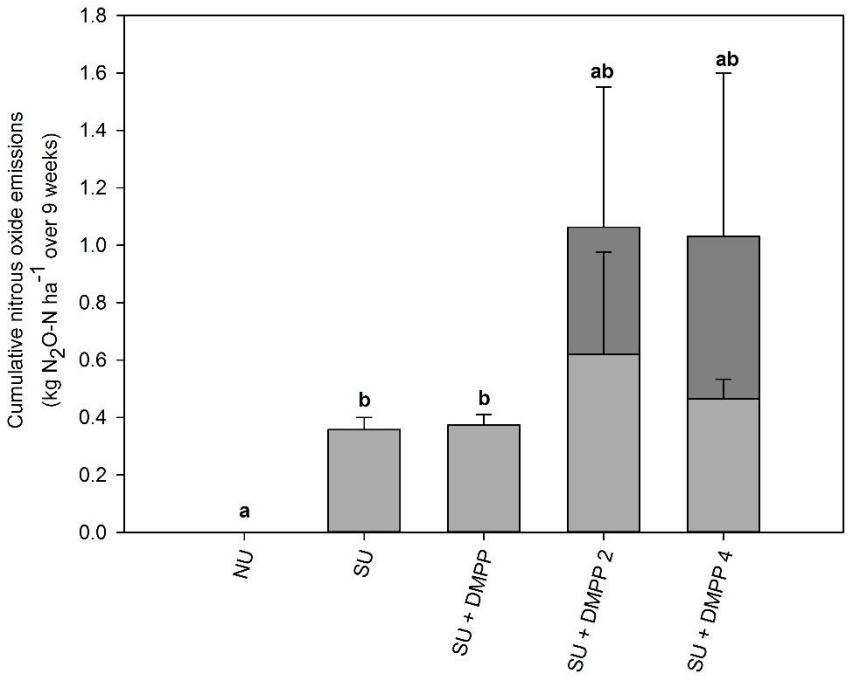

Figure 7.3. Cumulative nitrous oxide emissions, over 9 weeks post urine and DMPP applications to a Eutric Cambisol. Light grey bars represent means where *n* = 3, dark grey bars represent means where *n* = 2, with the high emitting replicates removed. Error bars denote SEM and different lower case letters represent significant differences (Welch's test, with Games-Howell pairwise comparisons) between treatments, where the same groupings were found both with and without outliers removed.

7.3.4 Diurnal nature of N_2O emissions

In order to investigate the diurnal nature of N_2O emissions, data were selected over the period from 08/08/2015 to 14/08/15. The 'high emitting' replicate from SU + DMPP 2 treatment was chosen for investigation, as it is unlikely that other factors e.g. available N and C, were limiting N_2O production during this period. The diurnal nature of N_2O emissions can be seen in Fig. 7.4, where N_2O emissions are shown to be out of phase with soil temperature, which may indicate a lag phase between soil temperature and the activity of N_2O producing microorganisms. The maximum N_2O emissions over this diurnal cycle generally occurred

between ca. 00:30 and 06:30 (hh:mm), and the minimum emissions in the diurnal cycle occurred between ca. 15:30 and 21:30 (hh:mm). The maximum soil temperature over the period of observation shown in Fig. 7.3 was 26 ºC, and the minimum soil temperature was 12 ºC. The highest soil temperatures generally occurred at around 13:30 (hh:mm), however the minimum temperature occurred anywhere between 00:00 and 06:00 (hh:mm).

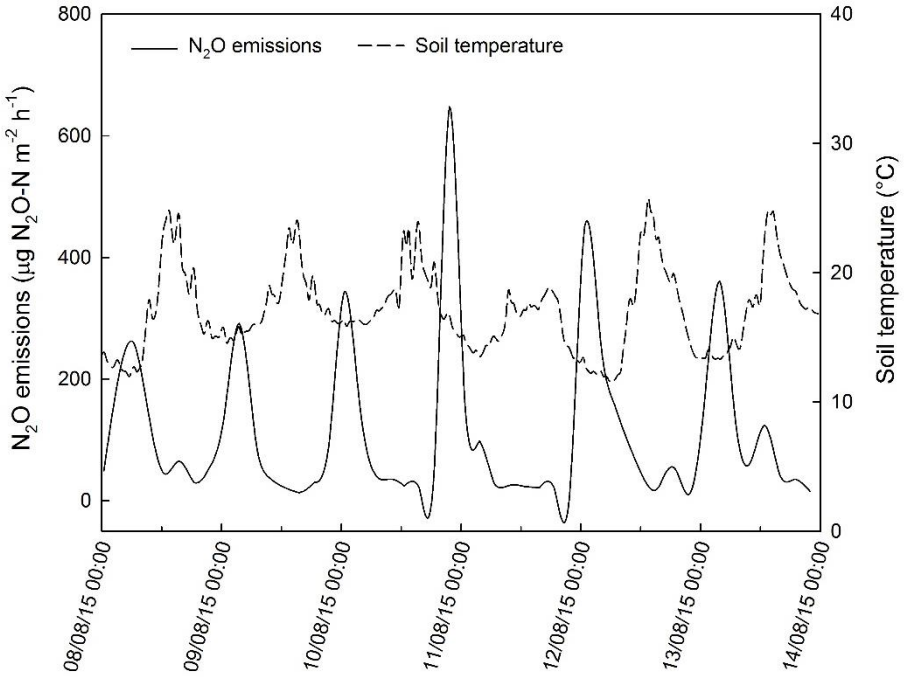

Figure 7.4. Diurnal nature of N_2O emissions and soil temperature over 6 days. The solid line represents the measured N_2O flux from a single high emitting replicate from SU + DMPP 2 treatment, and the dashed line is soil temperature as measured by the Acclima SDI-12 combined temperature and moisture sensors in the chamber. The date/time format along the x-axis is expressed in dd/mm/yy hh:mm.

7.3.5 Mineral N dynamics

The extractable NH_4^+ and NO_3^- from soil cores in replicated plots can be seen in Figure 7.5a and b, respectively, and the soil solution NH_4^+ and NO_3^- from Rhizon samples can be seen in Figure 7.5c and d, respectively. Prior to urine application, no significant differences ($p >$

0.05) in extractable NH_4^+ concentrations were observed between treatments. On the day of urine application (27/07/15), all extractable NH_4^+ concentrations were greater than the control ($p < 0.01$), with no further differences between treatments. On the second day following urine application, extractable NH_4^+ was only higher ($p < 0.05$) in the SU treatment in comparison to the control. Four days following urine application the extractable NH_4^+ was greater in all DMPP containing treatments ($p < 0.05$) compared to the control, but the SU treatment had returned to control levels ($p > 0.05$), indicating DMPP applications were delaying nitrification. One week following urine application the SU and SU + DMPP 4 treatments had returned to control levels ($p > 0.05$), however, the SU + DMPP and SU + DMPP 2 treatments had higher extractable NH_4^+ concentrations in comparison to the control ($p < 0.05$). This indicates that the effect of DMPP was shorter lived in the SU + DMPP 4, compared to SU + DMPP 2 and SU + DMPP treatments. No differences were observed in extractable NH_4^+ between treatments 10 days after urine application. Two weeks following urine application SU + DMPP had a higher extractable NH_4^+ content compared to the control ($p < 0.05$), and after 3 weeks extractable NH_4^+ was greater in SU + DMPP and the SU + DMPP 2 compared to the control ($p < 0.05$). No further differences in extractable NH_4^+ concentrations were observed beyond three weeks following urine application ($p > 0.05$).

No differences were observed in extractable NO_3^- concentrations between treatments prior to, on the day of urine application or two and four days following urine application ($p > 0.05$). After one week, however, all urine containing treatments had a higher extractable NO_3^- content in comparison to NU ($p < 0.001$). Ten days following urine application the extractable NO_3^- concentrations were still greater than NU in all urine containing treatments. Here, the SU had a higher extractable NO_3^- concentration than the SU + DMPP treatment, but not the SU + DMPP 2 or SU + DMPP 4 treatments, indicating a delay in nitrification when DMPP was applied at the same time as the urine only. Two weeks after urine application, extractable NO_3^-

was greater in all urine containing treatments compared to NU, but no other differences were detected beyond this date ($p > 0.05$).

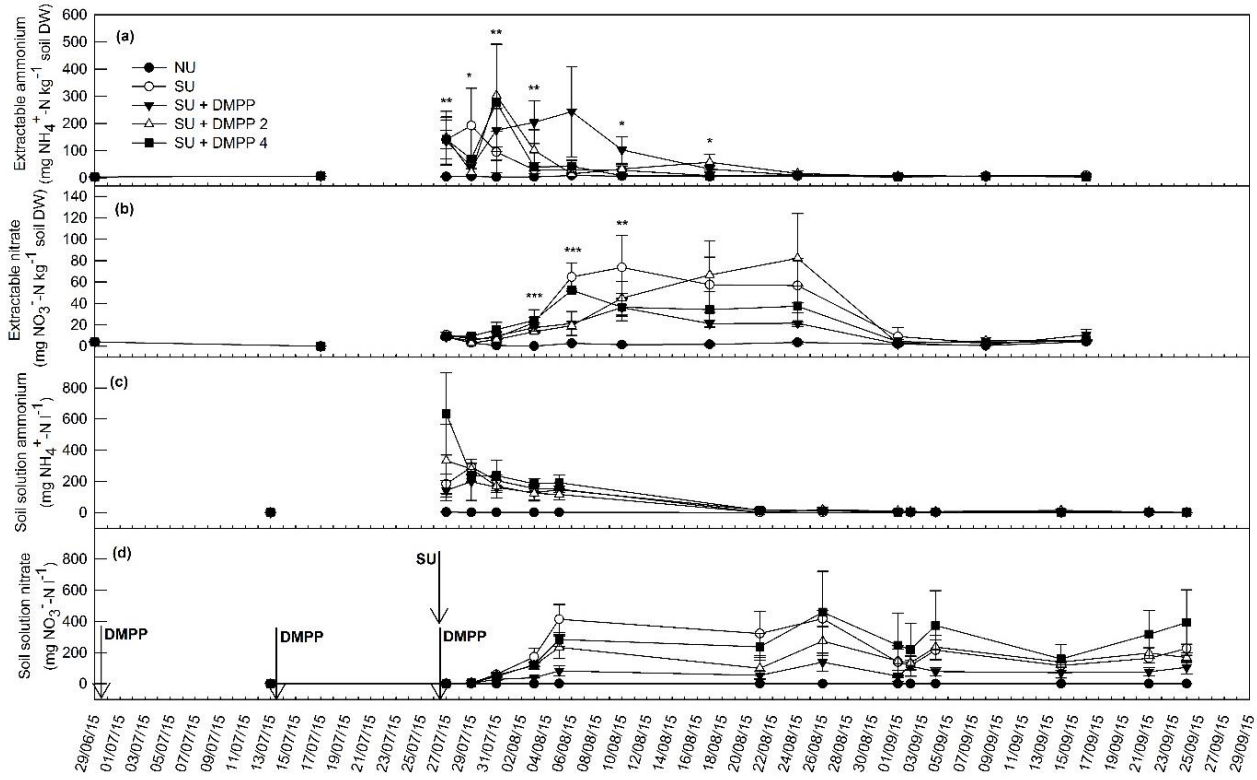

Figure 7.5. Mineral nitrogen dynamics during the course of the field trial. Extractable ammonium-N can be seen in panel a) and extractable nitrate-N in panel b), which were measured on extracted soils from replicated plots (outside chambers). The soil solution ammonium-N is shown in panel c) and soil solution nitrate-N in panel d), which were measured from Rhizon samples located within the chambers. The figure legend applies to all panels, symbols represent means ($n = 3$), and error bars denote SEM. Arrows in panel d) represent timings of sheep urine (SU) and/or DMPP applications to the respective treatments. Asterisks above symbols represent level of significant differences (ANOVA) and was conducted for data in panels a) and b) only. The date format along the x-axis is dd/mm/yy.

Rhizon soil solution samples were only obtained when soil conditions were moist enough, therefore n varied one each sample date, depending on whether sufficient sample was obtained for analysis. Soil solution NH_4^+ concentrations in the treatments containing sheep urine appeared to return to control values at a similar time to the soil extracts. The soil solution NO_3^- concentrations increased steadily during the 9 days following urine application. At the point of 9 days following urine application, the soil solution NO_3^- followed the numerical trend C < SU + DMPP < SU + DMPP 2 < SU + DMPP 4 < SU, which indicates DMPP delayed nitrification rates compared to sheep urine alone, but was less effective with the longer the time since DMPP application. Notably, the soil solution NO_3^- concentrations were numerically higher than NU for a longer period in comparison to the extractable NO_3^- data, indicating a difference in N processing rates between duplicated plots and the chambers.

7.3.5 Dissolved organic C and N dynamics

The total extractable N and dissolved organic C during the course of the field trial can be seen in Fig. 7.6a and b, respectively; the total N and dissolved organic C measured in the soil solution samples can be seen in Fig. 7.6c and d, respectively. Differences between total extractable N were only found at one sampling point following urine application to the plots, this was at two weeks following urine application where the SU + DMPP extractable N was greater than the control ($p < 0.05$). Similarly, not many differences in extractable dissolved organic C were found between the treatments at the different sampling points. Here, the only differences found were two days following urine application, where SU and SU + DMPP 2 had higher levels of extractable dissolved organic C compared to the control ($p < 0.05$).

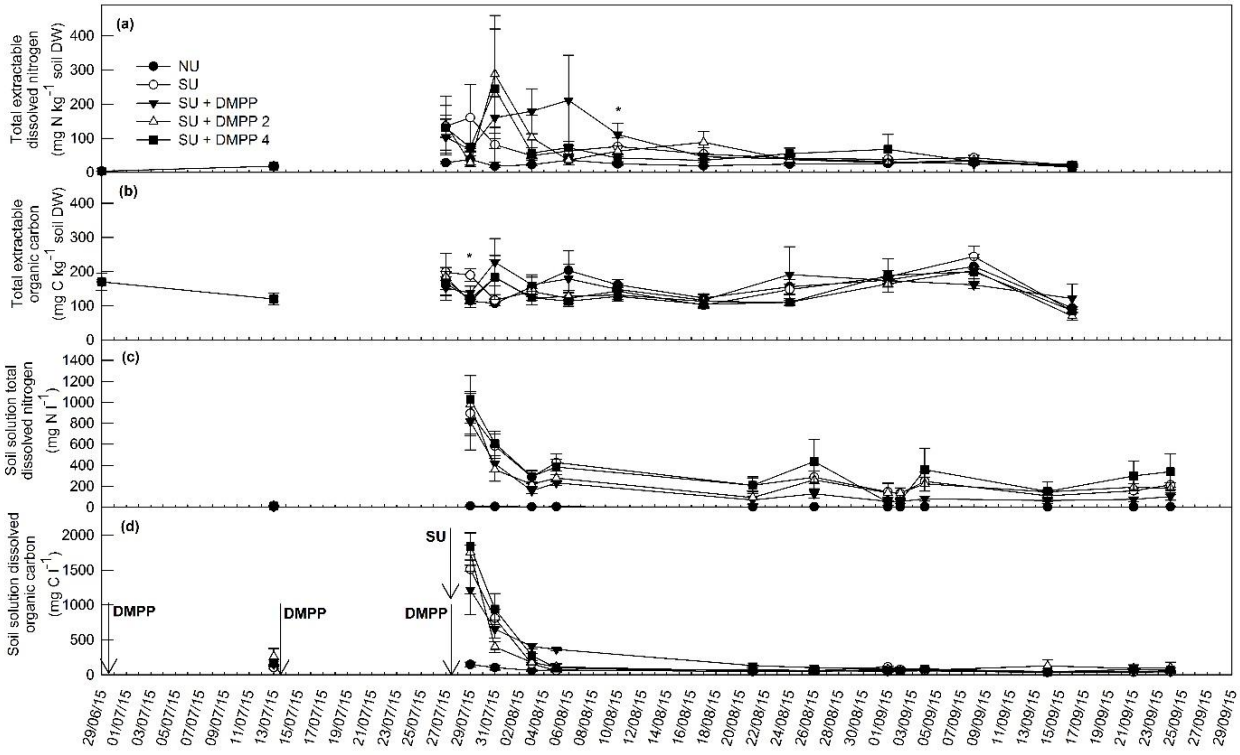

Figure 7.6. Total extractable and soil solution dissolved N and C dynamics. Total extractable dissolved N is displayed in panel a) and total extractable dissolved organic C is displayed in panel b), which were measured on extracted soils from replicated plots (outside chambers). Soil solution dissolved N is displayed in panel c) and soil solution dissolved organic C is displayed in panel d), which were measured using Rhizon samplers located within the chambers. The figure legend applies to all panels, symbols represent means (n = 3), and error bars denote SEM. Arrows in panel d) represent timings of sheep urine (SU) and/or DMPP applications to the respective treatments. Asterisks above symbols represent level of significant differences (ANOVA) and was conducted for data in panels a) and b) only. The date along the x-axis is expressed in dd/mm/yy.

The soil solution N increased following urine application, and remained numerically higher than the control until the end of the study, which reflected the remaining NO_3^- in the soil solution of the chamber plots. In the two chambers which emitted the highest amounts of

N_2O, it was found that the soil solution dissolved organic C concentration was ca. 2 and 4 times higher than the other samples collected across the plots, prior to urine application. The soil solution dissolved organic C concentration increased following urine application and treatments appeared to decline at similar rates. No observable differences in soil solution dissolved organic C were found between treatments after ca. 3 weeks following urine deposition.

7.3.6 Pasture biomass and foliar N content

The foliar N content measured at block level (n = 3), was 3.5 ± 0.5% prior to urine application. No significant differences ($p > 0.05$) in foliar N content were observed between treatments 3 weeks following urine application, where the mean foliar N content across all treatments was 3.4 ± 0.2%. The urine containing treatments all had a significantly greater pasture foliar N content in comparison to the control ($p < 0.01$) at 6 and 9 weeks following urine application, where the mean foliar N content of all the urine containing treatments was 4.3 ± 0.1% and 4.6 ± 0.2%, compared to the control value of 3.2 ± 0.2% and 3.0 ± 0.4%, respectively. No significant differences ($p > 0.05$) were found for pasture biomass in the plots at 3, 6 or 9 weeks following urine application, where the mean pasture biomass across all samples were 0.47 ± 0.2, 0.51 ± 0.03 and 0.29 ± 0.04 t DM ha^{-1}, respectively.

7.4 Discussion

7.4.1 Effect of DMPP on sheep urine patch N_2O emissions and mineral N dynamics

In this study, a liquid DMPP application (1 kg ha^{-1}) did not reduce cumulative N_2O emissions from sheep urine patches under UK summer conditions; this was true when DMPP was applied 4 weeks before, 2 weeks before and even when applied on the same day as the sheep urine. Whilst no significant effect of DMPP was found for cumulative N_2O emissions,

there was an observable effect of the nitrification inhibitor based on temporal trends in the N_2O fluxes and mineral N data. When considering the SU + DMPP treatment, the initial N_2O peak following urine application was much reduced in comparison to the SU only treatment, however, a broader, shallower peak towards the end of the measurement period was observed. In addition, the extractable NH_4^+ concentrations were higher in the SU + DMPP and SU + DMPP 2 treatments up to three weeks following urine application. This suggests that nitrification rates were delayed, with the effect being greater the shorter the time since DMPP application. Whilst some effect of DMPP was observable, this tended to delay, rather than reduce overall N_2O emissions in this study. The N application in the sheep urine patches was relatively high at 725 kg N ha^{-1}, yet Di et al. (2012) found DMPP reduced N_2O emissions by 66% from cattle urine N applications as high as 1000 kg N ha^{-1}, although a higher DMPP application of 5 kg ha^{-1} was used in this study.

Reasons for not observing an effect of DMPP on cumulative N_2O emissions could be because of the high soil temperatures, which may have resulted in rapid DMPP degradation by microbial activity. Menéndez et al. (2012) and Vasquez et al. (unpublished), however, found no effect of increasing temperature between 10 and 20 ºC on DMPP degradation, which represents the typical range of soil temperatures observed in this field trial. The soil water content has also been identified as an important parameter influencing the persistence of this molecule in soil, where a lower water content (and hence greater oxygen content) may increase oxidation of the molecule (Chen et al., 2010; Menéndez et al., 2012). In this study the maximum observed water-filled pore space was 69%, but more often it remained between 20 and 40%, which renders depletion of DMPP via oxidation a possible degradation pathway. Further depletion of DMPP from the soil solution could have occurred due to immobilisation within non-target microbial biomass or canopy interception of the applied inhibitor.

Another possible reason for the lack of effect of DMPP on cumulative N_2O emissions could be inefficient mixing with the soil solution and urine-N. Given that the DMPP was applied under rainfall conditions in the treatments SU + DMPP and SU + DMPP 2, and that there was an observable effect of DMPP on nitrification rates and the temporal nature of N_2O fluxes in SU + DMPP treatment, it would suggest that the inhibitor at least partially mixed with the urine derived N. The DMPP applied 4 weeks before urine application, however, may have experienced more degradation or pasture canopy interception during application, as it was not applied during a rainfall event. Further research based on different DMPP application methods is required, e.g. investigating whether DMPP would have been more effective if mixed with the urine prior to application to soil. Comparatively more research has been conducted on different application methods of DCD in comparison to DMPP (e.g. Ledgard et al., 2008; Minet et al., 2013; Wakelin et al., 2013; Luo et al., 2015). This may be due to the original commercial development of DMPP as a fertiliser coating (Pasda et al., 2001; Zerulla et al., 2001), rather than for its use on urine-influenced soils.

7.4.3 Spatial variability in N_2O fluxes

N_2O emissions from agricultural soils are notoriously variable, both in time and space (Hénault et al., 2012), and this study proved to be no exception. Notably the SU and the SU + DMPP treatments did not have as high a variability in N_2O emissions as the SU + DMPP 2 and SU + DMPP 4 treatments. Due to this we can conclude with more confidence that DMPP had no effect on cumulative N_2O emissions when applied at the same time as the urine under these experimental conditions. Bearing this in mind, it is unlikely that the DMPP would have had an appreciable effect on emissions when DMPP was applied weeks before the urine, even if the data had not suffered from the large spatial variability.

Whilst a high temporal variability was observed, the automated chamber system captured this variability, thereby reducing bias in emission calculations due to sampling time and frequency, which can be an artefact of manually sampled static chamber based campaigns e.g. Yamulki et al. (2001), Scheer et al. (2014). The N_2O emissions in this study were also, however, highly spatially variable. Here, one replicate in each of the treatments SU + DMPP 2 and SU + DMPP 4 demonstrated a marked difference to their respective replicates. Future studies should consider maximising the number of replicates in order to capture spatial variability in N_2O emissions, when using an automated chamber based system. This will potentially result in a having to select fewer treatments for investigation, however, would be appropriate where emission factors are to be measured for inventory purposes.

Prior to urine application, the soil solution samples from inside the 'high emitting' chambers had 2 (242 mg C l^{-1}) and 4 (489 mg C l^{-1}) times the amount of dissolved organic C compared to the mean of the remaining soil solution samples (125 ± 10 mg C l^{-1}; mean ± SEM; n = 10). The spatial heterogeneity in dissolved organic C may have been caused by several reasons, including root exudation in the 'rhizosphere', litter decomposition and root death in the 'detritusphere' and labile and recalcitrant organic material passed through the hind gut of soil organisms or faeces of larger organisms in 'biopores', which are recognised as hotspots of microbial activity (Kuzyakov and Blagodatskaya, 2015). Urine patches themselves are also considered hotspots of microbial activity. As the plots in this study were fenced off several months prior to the simulated urine applications, the possibility of the high dissolved organic C arising from a previous urine deposition is unlikely. Whatever the origin of the labile C in these plots, we believe the overlapping of two unique hotspots occurred. The presence of greater amounts of labile C in the two high emitting chambers prior to urine application may have resulted in an increased abundance and activity of soil microorganisms (e.g. nitrifiers and denitrifiers), compared to the soil enclosed by the other chambers. The addition of more labile

C and N in the form of urine, may then have led to rapid N turnover and increased N_2O emissions from these plots. Alternatively, DMPP could have provided a C source in itself, where uneven applications within the chambers may have resulted in a large variability in emissions.

7.4.2 N_2O emission factors

No differences were found for N_2O emission factors between treatments. The mean N_2O emission factor across all treatments (n = 12) was 0.81 ± 0.22% of the applied N. The lowest emission factor recorded was 0.30% of the applied N which occurred in the SU + DMPP 2 treatment, and the highest emission factor was 2.49% of the applied N which occurred in the SU + DMPP 4 treatment. In general, emission factors were lower than the current default IPCC emission factor of 1% of the applied N for sheep excreta (IPCC, 2007), which is consistent with reported emission factors being lower under spring-summer conditions in comparison to autumn-winter (Allen et al., 1996). Nevertheless, the overlapping of nutrient hotspots more than doubled the default IPCC emission factor for sheep urine, where emission factors of 2.24 and 2.49% of the applied N occurred. The measurement period of this study was shorter than the recommended emission factor measurement period of 1 year and it did not include a measurement of emissions from the dung fraction of excreta, therefore, care must be taken when considering these emission factor values.

7.4.4 Diurnal nature of N_2O fluxes

The existence of diurnal trends in N_2O flux data has been identified in several studies (e.g. Thomson et al., 1997; Yamulki et al., 2000; Yamulki et al., 2001; Hyde et al. 2005a; Hyde et al., 2005b; Hatch et al. 2005; Alves et al., 2012; Scheer et al., 2014). Scheer et al. (2014) found the diurnal nature of N_2O fluxes appeared to be greatest when the mineral N and moisture

were non-limiting. This appeared to be true in our study, and the diurnal trend was also strongest in the chambers where urine was applied to a dissolved organic C hotspot. Our study shows that maximum N_2O fluxes occurred at night time and early morning, where the diurnal nature of fluxes was out of phase with soil temperature. A similar phenomenon was observed in other temperate grassland systems where cattle urine (Yamulki et al., 2000; Yamulki et al., 2001) and ammonium-nitrate fertilizer (Hyde et al., 2005) were the N sources. In the studies by Yamulki et al. (2000) and Yamulki et al. (2001), maximum N_2O fluxes occurred between the late afternoon and early morning and Hyde et al. (2005a) observed minimum N_2O fluxes at mid-day and maximum fluxes between 20:00 and 22:00 (hh:mm). However, other studies have also shown soil N_2O flux and soil temperature to be closer in phase with each other, e.g. Hatch et al. (2005) showed a lag time of only 30 minutes between maximum temperature and N_2O fluxes, although this study was conducted under controlled conditions, on repacked soil cores, in the absence of plants and with slurry as the N source. In the study by Scheer et al. (2014), maximum emissions occurred between 12:00 and 15:00 (hh:mm) and minimum emissions occurred between 04:00 and 07:00 (hh:mm); in this study, the diurnal fluxes were assessed in a treatment containing fertilizer application, after the incorporation of broccoli residues in an intensive broccoli production site in a sub-tropical climate.

The observed variation could be due to the fact that N_2O emissions are dependent on several factors (not just soil temperature), which may interact in different ways to produce different timings of minimum and maximum flux. In addition, the solubility and microbial consumption of O_2 increases alongside temperature, which may lead to reductions in N_2O due to the occurrence of complete denitrification (Yamulki et al., 2000). The pattern of N_2O production and consumption across soil depth may also vary with temperature, where maximum and minimum temperatures would occur later at soil depth. Another possible reason is that greater compaction could result in slower gas diffusivity (Ball et al., 1999; Sitaula et al.,

2000) from the source of N_2O to the chamber headspace i.e. a shorter lag between maximum temperature and maximum N_2O flux was found in the sieved and repacked soils of Hatch et al. (2005), as well as soils which had been subjected to rotary hoeing during incorporation of vegetable residues in Scheer et al. (2014). This area of study requires further research as it has implications for recommendations of the best time to sample N_2O emissions over a 24 h period to reflect the average daily fluxes. It also supports the use of methods with a high temporal resolution for monitoring N_2O emissions, where possible.

7.4.5 Destructive vs. non-destructive soil sampling for measuring soil mineral N pools

In this study differences were found between the mineral N dynamics as measured by taking soil cores from duplicate urine patches (destructive) and as measured in the soil solution of the chambers (non-destructive). The soil solution NO_3^- concentrations in the urine treated chambers remained higher than the control for a longer period than the extractable NO_3^-, therefore, further N_2O emissions could have occurred beyond the duration of this study. The trends in soil solution and soil extractable NO_3^- concentrations are more indicative of whether N processing rates were occurring at similar rates in the chambers, than comparing soil solution NH_4^+ and soil extractable NH_4^+. Provided N processing rates are occurring equally, the soil solution NO_3^- should follow similar temporal trends to the extractable NO_3^-, due to limited sorption occurring for NO_3^-. Conversely, the soil solution NH_4^+ data represents the pool of NH_4^+ that was not bound to exchange sites, therefore, comparisons between the processing rates of NH_4^+ in the soil solution and the soil extracts cannot be made, as they represent different pools. Nevertheless, soil solution NH_4^+ concentrations in the treatments containing sheep urine appeared to return to control values at a similar time to the soil extracts.

The differences in N processing found between the duplicated urine patches may reflect artefacts of coring the duplicated urine patches. This may have influenced the aeration and

water infiltration dynamics in these areas. Conversely, the chambers may have caused more shading and/or interception of rainfall in comparison to the duplicated plots. The difference in N processing rates highlights the need for the development and use of non-destructive sampling techniques for use inside chambers. Rhizon samplers were useful in this study for determining differences, which would not have been observed from only taking soil cores from duplicated urine patches. Limitations of the Rhizons were that successful sample collection depended on sufficient soil moisture, which sometimes resulted in a variable number of replicates on different sample dates. Deploying multiple Rhizons within a urine patch could potentially overcome the issue of the occasional small sample volume. Nevertheless, Rhizon samplers would be ineffective under extended dry periods unless the soil water content is modified artificially, which is not recommended as modification of the soil water content may also modify N processing rates.

7.5 Conclusions

A recent review by Gilsanz et al. (2016), highlights the paucity of studies regarding DMPP efficacy in reducing N_2O emissions, in comparison to the well-researched inhibitor DCD. This is especially true of urine-influenced soils, where this study contributes new information for DMPP efficacy in sheep urine patches, under UK summer conditions. In this study, DMPP influenced the dynamics of N processing rates, but had no effect on overall cumulative N_2O emissions or emission factors. Further research should be conducted to determine whether DMPP is effective in livestock grazed pastures at a range of urine-N applications, under different seasonal conditions and with a range of nitrification inhibitor delivery methods to the soil.

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Chapter 8

Discussion and recommendations for future research

8.1 Introduction

Detailed discussion of the results from the experiments in this Thesis are described in each experimental Chapter. In this Chapter, the experimental work presented in Chapters 3-7 is summarised and discussed in relation to the common themes within the Thesis, the results are then discussed in relation to the initial objectives, and in terms of the wider implications of the results. An assessment is made of how future mitigation strategies and research should be prioritised in order to achieve enhanced sustainability and productivity in livestock grazed grasslands.

8.2 General discussion

This section discusses the results in terms of common themes within the Thesis. This Thesis consists of five experimental chapters where sheep urine (real or artificial) and/or nitrification inhibitors (DCD and DMPP) are applied to differing soils, with or without the presence of plants, in either laboratory or field settings.

Real sheep urine was applied to soils within Chapters 3, 5 and 7, and artificial urine was used in Chapter 4. The collected sheep urine samples used in Chapter 3 had a lower N concentration (3.6 g N l^{-1}) than that of the urine samples collected and used for Chapter 7 (14.5 g N l^{-1}). The urine patch N loading rate used in Chapter 3 equated to around 200 kg N ha^{-1}, whereas the N loading rate in the field trial of Chapter 7 was 725 kg N ha^{-1}. Artificial urine was made up at two concentrations (4 g N l^{-1} and 16 g N l^{-1}) and applied at two N loading rates (equivalent to 200 kg N ha^{-1} and 800 kg N ha^{-1}) for use in Chapter 4, which were similar to the measured range of urine N concentrations found in Chapters 3 and 7. The observed range in urine N concentration supports other findings of a wide range in urinary N concentrations of grazing animlas, despite similarities in the diet (*Lolium perenne* L. dominated sward) fed to the animals and the breed under investigation (Welsh Mountain ewes).

The nitrification inhibitor DCD was utilised in Chapters 5 and 6 and DMPP was utilised in Chapters 5 and 7. In Chapters 5 and 6 it was shown that DCD was bioavailable to both soil microorganisms and plants, respectively. While DMPP was also acquired by soil microorganisms, it appeared to be less available to soil microbes in comparison to DCD, which may explain the slower degradation rates of DMPP in comparison to DCD. Direct comparisons of the efficacy of DCD and DMPP were not made, although factors influencing their vertical mobilities were compared in Chapter 5. Both nitrification inhibitors behaved in different ways in contrasting soils, suggesting that differences in their efficacy in contrasting soils may also occur. The nitrification inhibitor DMPP was chosen for use in the final field trial (Chapter 7) as fewer studies had been conducted using this inhibitor to target urine patches in the soil compared to DCD, and due to the fact that the recent suspension of the sale and use of DCD may prompt the use of alternative nitrification inhibitors within pasture soils. DMPP was found to be ineffective at reducing emissions of N_2O from the Eutric Cambisol, under the conditions of field trial in Chapter 7. The results of the studies on nitrification inhibitors suggest that they are not a one-stop solution for reducing N_2O emissions from urine patches. Further work is required to optimise their efficacy in a cost-effective manner e.g. by using at high risk times, in high risk areas or by using technologies to apply directly to urine patches in the soil.

A common soil type (Eutric Cambisol; sandy clay loam) was utilised in Chapters 3, 4, 5 and 7; a sandy loam textured Eutric Cambisol was used in Chapter 5; and a Histosol was used in Chapters 5 and 6, to collectively provide some contrasting soil conditions. The Cambisols are one of the most common soil types based on land area (covering some 1.5 billion ha), and are widely used for food production (FAO, 2001), therefore, these soils represent an important area of study. Sheep urine was applied to a Eutric Cambisol in Chapters 3, 4 and 7. The maximum extractable NO_3^- following urine application to these soils were similar in the field trials (Chapter 4 and 7; ca. 100 mg NO_3^--N kg^{-1} soil DW), but was higher in the laboratory

study (Chapter 3 ca. 270 mg NO_3^--N kg^{-1} soil DW) which is likely to be an effect of the absence of plants in this study. The time taken for NO_3^- to peak in these soils following urine application was similar, ranging from ca. 20-28 days. The extractable dissolved organic C following urine application to the Eutric Cambisol was similar in these three studies, ranging between 100-200 mg C kg^{-1} soil DW. This suggests that C availability is not limited in these soils, however, this was measuring the extractable pool, where the organic C may be bound to soil particles and potentially unavailable to the soil microbes. The soil solution dissolved organic C is more indicative of what is potentially bioavailable following urine deposition, and this was measured in both Chapters 4 and 7. These results both show a sharp increase and decline of dissolvd organic C immediately after urine application, which also peaked at similar values (ca. 2000 mg C l^{-1}), despite the fact that artificial and real urine were used in the different studies. This suggests that the artificial urine may have adequately represented the composition of real urine. This peak of organic C was short-lived, however, lasting a period of just under a week in Chapter 4 and just over a week in Chapter 7 (this generally coincided with peak in N_2O emissions following urine application). This suggests that the labile C applied to or created (e.g. priming or solubilisation of organic matter) within the urine patch is rapidly consumed, where conditions then return to similar values as measured prior to urine application. The N_2O emissions varied quite widely from this soil type, with the highest emissions occurring under the laboratory study. This may have been expected as conditions of moisture were maintained at high values (70% WFPS) throughout the incubation and there were no plants competing for the mineral N within the soil. The major N_2O emission period lasted for around 20 days after urine application in Chapters 3 and 4, but lasted for a longer period in Chapter 7 (ca. 60 days after urine application, mainly in the replicates which emitted high amounts of N_2O). It is recognised that emissions could have been monitored for longer time periods e.g. to calculate emission factors 1 year of measurements are recommended. Levels of NO_3^- had returned to

control values by the end of the study period in Chapter 3 and 7, however, there was still some NO_3- available at the end of the study in Chapter 4, where N_2O emissions may have still occurred following rainfall events.

In Chapters 4, 6 and 7, pasture plants were present within the studies, however, no plants were present in Chapters 3 and 5. The inclusion of plants within studies of the urine patch is important due to the role plants play in regulating substrate availability in the rhizosphere. The absence of plants in Chapter 3 is likely to have resulted in an over-estimation of N_2O emissions, as it is likely that more N would have been available in the soil for N_2O producing processes. It may have also increased lateral diffusion of N, due to higher concentrations within the centre of the urine patch, creating a larger concentration gradient for diffusion. While Chapter 3 examined the urine patch diffusional area, a consideration of the pasture response area could also be important for accurately monitoring N_2O emissions from urine patches within chambers. The chamber walls may prevent the growth of adjacent plant roots into the urine patch, resulting in less of the available N being incorporated into plant tissues. The presence of plant roots may have altered the flow pathways of urine, DCD and DMPP in the soil columns of Chapter 5. Rhizosphere microorganisms may have played a greater role in the immobilisation of DCD and DMPP, if plants roots and their associated microbial communities were represented in the soil columns. This may have overestimated the vertical movement of the inhibitors in the studied soils.

Three of the experimental Chapters were laboratory studies (Chapters 3, 5 and 6), which are useful for understanding mechanistic processes; nevertheless, these studies are limited in what can be concluded from them, due to dissimilarities between laboratory and field conditions. In Chapter 3, 5 and 6 sieved soils were used; while this creates homogeneous conditions, it may misrepresent conditions in the field. Conditions which are modified include the breakdown of aggregate structures affecting soil porosity and bulk density, the removal of

stones and roots which can affect preferential flow pathways, homogenisation of the soil which removes any natural depth distribution of microorganisms and the potential breakage of fungal hyphae which may stimulate the release of nutrients into the soil. This has to be considered when interpreting the results of the laboratory studies in this Thesis, and extrapolations to field conditions need to be considered with care. The sieved soils were re-packed to a depth of 5 cm in Chapter 3 and to a soil column depth of 15 cm in Chapter 5. The use of shallow trays in Chapter 3 may have promoted lateral diffusion of solutes, while the use of small soil columns in Chapter 5 may have promoted vertical movement of urine and NI. Using intact pasture blocks or pasture soil cores, with a greater soil depth than 5 cm would be preferential for future laboratory studies, as many of the soil conditions would remain unchanged, yet environmental parameters such as moisture and temperature could be controlled.

Two of the experimental chapters were field based studies (Chapters 4 and 7). The data gathered from field trials are generally more realistic, although trends can be difficult to interpret due to confounding factors which can be present under natural conditions. A large variability was observed in many of the measured soil parameters and N_2O emissions, which reflects the inherent spatial variability present within pasture soils. While the soil type and urinary N concentrations deposited were fairly similar in the two field trials, N_2O emissions differed largely on the two separate occasions. This is likely to be due to the different climatic regimes observed during the trials, where one was comparatively warmer and drier (Chapter 4) and the other was cooler and wetter (Chapter 7).

8.3 What implications do variations in urine patch and environmental parameters have on the upscaling of N_2O emissions from grazed grasslands?

The first objective was to determine how a) sheep urine patch parameters and b) environmental parameters influence N_2O emissions from grassland soils. In Chapter 4, the

effect of urine patch size and N concentration within urine patches was assessed. From this study it was concluded that increasing urine N concentration increased cumulative N_2O emissions, but not the N_2O emission factor. An effect of patch size was also observed, when holding N application rate constant, where four smaller patches emitted more N_2O than a single large urine patch, at an equivalent N loading rate of 800 kg N ha^{-1}. There is a paucity of data regarding urination frequency, volume and N concentration under differing diets, management strategies and environmental conditions. The importance of considering the frequency and concentration distribution of urine events for modelling N losses from grazed pastures is becoming more apparent (Li et al., 2012). Advances in sensor-based technology for monitoring urine events *in situ* will be most useful in the future (e.g. Betteridge et al., 2010, 2013), to feed into models, and to test management and mitigation measures under more natural conditions (i.e. compared to housing animals and subsequent urine collection, which may influence the urine composition).

In Chapter 3, changes in N_2O emissions at the urine patch-soil interface were assessed, under controlled conditions. The most important driving variables for N_2O emissions under controlled conditions were water-filled pore space (WFPS), redox potential and total extractable N content. In Chapters 4 and 7, peaks in N_2O emissions also appeared alongside rainfall events. Under the warm and dry conditions in the field trial of Chapter 4, the N_2O emissions from sheep urine patches over 10 weeks were ten times lower than the IPCC default emission factor for N_2O from sheep excreta. In the late summer urine application there was more rainfall and emission factors were generally closer to that of the default (0.80% of applied N), however, emissions were still not measured for a whole year, and further emissions could have occurred beyond the period measured in this study. Scaling up from individual chamber-based urine patch fluxes to field scale fluxes can cause uncertainty for livestock based systems. Typically, livestock have to be excluded from plots with chambers, yet, the presence of

livestock is likely to alter emissions e.g. no consideration of compaction caused by hooves or overlapping of urine patches (de Klein et al., 2006; Pleasants et al., 2007; Cichota et al., 2013). The use of collars which are low lying to the ground would allow livestock to be present within a chamber study site. Upscaling of emissions would also use mean values for urine volume and frequency per head of livestock, yet, this may cause uncertainties due to differences in N processing rates of patches of different sizes.

The IPCC Tier 1 approach to calculating N_2O emission factors is too simplistic and does not consider the effect of soil type, management strategies or climatic variables on excretal emissions (de Klein et al., 2001; Skiba et al., 2012). A move to IPCC Tier 2 and 3 approaches is necessary to better constrain N_2O emission estimates from grasslands, where far more regional measurements of N_2O emissions from sheep excreta will be required. Disaggregation of N_2O emissions based on rainfall data and soil drainage class may be warranted, as variation between years may result in large biases of emission estimates (van der Weerden et al., 2012). In New Zealand, disaggregation of emission factors based on hill slopes has also been suggested to reflect the spatial difference in soil conditions and livestock resting behaviour in these areas, as most emission factor measurements are based on flat or lowland areas, which could represent an overestimation of N_2O emissions (Luo et al., 2013; Saggar et al., 2015). The effect of future climate scenarios are also important to consider, such as the expected increase in extreme weather events e.g. drought and flooding. Both could significantly affect N_2O emissions estimates, where emissions would be expected to be lower under drought conditions and higher under periods of heavy rainfall.

8.4 How should the chamber technique be deployed, to monitor N_2O emissions from urine patches?

The second objective was to determine how to accurately monitor N_2O emissions from urine patches, using the closed chamber technique. In Chapter 3, the importance of considering the urine patch diffusional area whilst monitoring N_2O emissions from urine patches was assessed. From the results of this study, it is recommended that, when calculating urine patch N_2O emission factors, extra room inside static chambers should be allowed for the diffusion of solutes into surrounding soil. Further work could be conducted here, by comparing the actual restriction effect posed by chamber walls in the field and assessing whether considering the pasture response area also influences N_2O emission measurements. Methods of measurements which do not disturb soil conditions or impact on the diffusion of solutes could be utilised, such as eddy covariance methods, or the use of sub-surface gas probes (Li and Kelliher, 2005) which do not rely on excluding stock from an area, which can influence N_2O emission e.g. by compaction with hooves. In addition, field validation of diffusion coefficient calculations for urine constituents should be conducted, where this approach could be used to estimate chamber size requirements to cover a urine patch.

In Chapter 4, an assessment of the headspace sampling frequency from static chambers following urine application was made. From this, it is recommended that where resources are available, sampling as close to daily as possible between 10 am and 12 noon, would closely resemble the fluxes as measured by an automated system. In Chapter 7, the diurnal nature of N_2O fluxes were examined, and maximum fluxes were observed during the night or early morning. It is likely that the diurnal pattern of N_2O emissions depends on land-use type, and site specific characteristics e.g. gas diffusivity through the soil profile. Therefore, where possible, an assessment of the diurnal variation in N_2O fluxes in the area of study should be

conducted prior to a manual sampling campaign. This will allow an assessment of the best time of day to sample to represent the average daily flux.

A large spatial variability in N_2O emissions was observed in the field trials of Chapters 4 and 7, despite the exclusion of livestock several months prior to treatment application. It is advised that replicate chambers should be maximised as much as resources allow, in order to capture this spatial variability. Automated chamber systems are generally limited by the number of available chambers, therefore, treatment numbers would need to be minimized to ensure sufficient replication, and hence an adequate spatial representation of N_2O emissions. It is also noted that this spatial variability can influence ancillary measurements taken to support N_2O emission factors. When taking ancillary measurements from duplicated urine patches, N processing rates can differ. Advances in non-destructive techniques to monitor urine patch characteristics will be important for future urine patch research.

8.5 Are nitrification inhibitors a suitable strategy for reducing N_2O emissions from grazed grasslands?

The third objective was to investigate the factors which influence nitrification inhibitor efficacy in ruminant urine-influenced soils and assess their potential as an N_2O mitigation strategy for sheep grazed grasslands. In Chapter 5, the vertical mobility and factors influencing vertical mobility (solubility, sorption/desorption, microbial uptake and degradation) of the nitrification inhibitors DCD and DMPP were assessed in three soils contrasting in texture and organic matter content, with and without the presence of sheep urine. It was found that the presence of sheep urine altered the depth distribution of both nitrification inhibitors, and increased desorption of the nitrification inhibitors, where this effect was soil and inhibitor type dependent. Sorption by soil was greater for DCD in comparison to DMPP in all tested soils and a large proportion of the applied inhibitors were immobilized by the microbial biomass.

Immobilization of nitrification inhibitors into non-target microbial biomass could equate to a large depletion of the bioavailable nitrification inhibitor pool. In this Chapter, it was shown that soil type is a regulator of the factors which can influence nitrification inhibitor efficacy. Further work is required to determine the comparative efficacy of DCD and DMPP in reducing nitrification rates and N_2O emissions from sheep urine patches, across a similar range of soil types. This may allow recommendations of which nitrification inhibitor, if any, are more effective under contrasting soil types.

In Chapter 6, the capacity for graminaceous plants to directly acquire DCD through roots, and to translocate this to shoots, was demonstrated. It was concluded that this is a potential vector for DCD entry into the food chain, and is one of the likely causes of the contamination of New Zealand milk products with DCD. The contamination of milk products from DCD-treated pasture has resulted in some consumer scepticism of nitrification inhibitor use, and alternatives are currently being sought (Bates et al., 2015). While DMPP is a different compound, and may not be as phytotoxic as DCD (e.g. leaf necrosis at 8 times the recommended application rate; Zerulla et al., 2001), it is still a synthetic compound which may also attract similar scepticism. A direct comparison of plant acquisition of both DCD and DMPP should be made, to confirm the same outcome will not occur with DMPP. Screening of plants capable of exuding biological nitrification inhibitors, through plant breeding, may prove to be a more fruitful approach (Subbarao et al., 2006).

The use of nitrification inhibitors in grazed pastures would be fairly quick to implement if a cost-effective delivery mechanisms can be developed, and they represent a useful strategy for improving nitrogen use efficiency in grazed pastures. However, the benefits associated with nitrification inhibitor use varies widely, suggesting benefits may not always outweigh the cost of application. This has implications for the widespread adoption of any mitigation strategy. In Chapter 7, DMPP applied to sheep urine patches under summer conditions was completely

ineffective at reducing cumulative N_2O emissions, even when applied at the same time as the urine. Further work is required to assess the reasons why it may not have been effective, where potentially higher application rates may have been required (which would also influence the cost of application) or temperatures may have been too high to render it effective.

Targeting urine patches within space and time is another major challenge associated with the effective use of nitrification inhibitors, and differing application methods will be a key area of research to bring this technology from a tried and tested concept to a widely adopted mitigation strategy. While orally administering synthetic nitrification inhibitors to ruminants has been examined, where large amounts of consumed inhibitor were excreted alongside the urine, this approach needs extensive ethical considerations. In particular, producers and consumers need assurance that there are no adverse effects of the inhibitors on grazing animal health and to ensure that no contamination would be present in milk or meat tissues destined for human consumption (Ledgard et al., 2008). As a wide range of compounds are able to inhibit nitrification, there may be compounds naturally present within urine or forage plants which could be utilised for targeted inhibitor-to-urine applications. Here, the screening of urine chemical constituents or feed constituents for nitrification inhibitor potential e.g. attempted study shown in Appendix 2, using a lux marked strain of *Ntrosomonas europeae*, could be a useful approach for novel nitrification inhibitor identification (Subbarao et al., 2006).

8.6 What alternative strategies are there for the mitigation of N_2O emissions from urine patches?

The mitigation of N_2O from urine patches is likely to require a combination of different strategies, to be most effective, which will need to be assessed by life cycle analysis and whole-farm system modelling approaches, in order to ensure the avoidance of pollution swapping (Eckard et al., 2010; Gerber et al., 2013; Schils et al., 2013). On-farm N_2O mitigation strategies

which have been utilised, tested or conceived can be broadly divided into plant, animal, urine, soil and management-based interventions, as shown in Figure 8.1. Nevertheless, some of these options would fit under several of the categories suggested here.

8.6.1 Plant-based mitigation strategies

Plant-based strategies to reduce urine patch N_2O emissions include the use of high-sugar ryegrass varieties in pastures. Such grasses have been shown to improve rumen N utilisation, potentially reducing N excreted in the urine (Moir et al., 2001; Edward et al., 2007). Pacheco et al. (2010) found urine N concentration to be inversely related to dietary cation-anion intake and soluble sugar content of differing forages, potentially reducing the N loading rate of individual urine events. Further field validation of potential effects on N_2O emissions and N utilisation by livestock are still required.

Condensed tannins are another potential plant-based strategy to reduce urine derived N_2O. Condensed tannin extracts are currently expensive, however, plant breeding may offer an approach to increase tannin levels in forage plants (Smith et al., 2010). Supplementation of condensed tannin extract has been shown to increase the proportion of N excreted in the faeces, and lower the N in the urine of both sheep (Carulla et al., 2005) and cattle (Misselbrook et al., 2005; Grainger et al., 2009). This is due to the complexation of condensed tannins with proteins in the rumen, which results in some protection against microbial degradation and can serve to

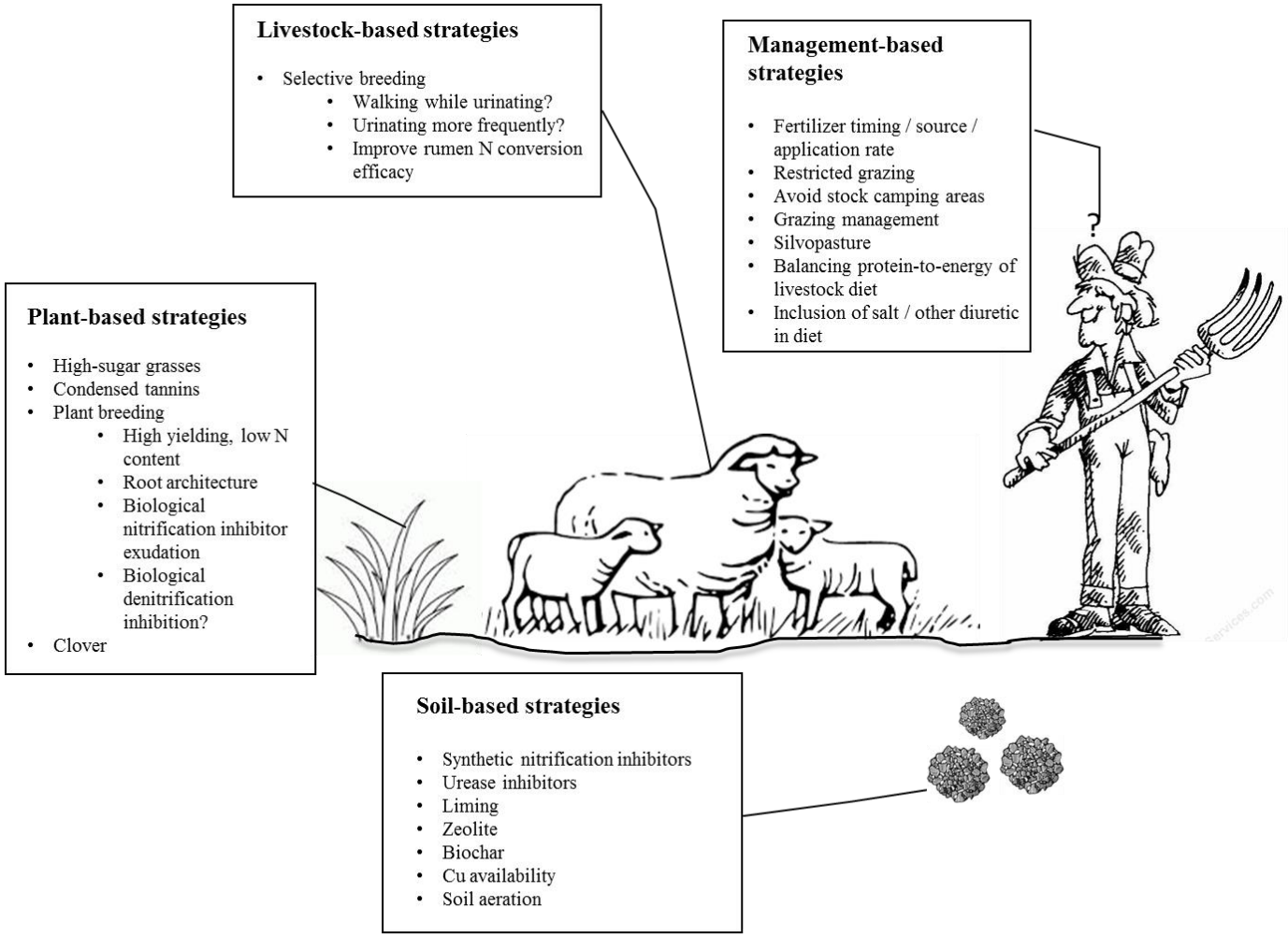

Figure 8.1 Strategies to reduce N losses from grazed pastures.

increase the absorption of amino acids in the abomasum or lower intestine, or the complex can be excreted in the faeces (Min et al., 2003; de Klein and Eckard, 2008; Smith, 2010). Other plant-based strategies which might reduce total N losses from grazed pastures is breeding plants which can intercept and acquire more urine or fertilizer N through differing root architecture, pasture with high yields but lower N contents and the inclusion of clover in pastures to increase biologically fixed N (Ledgard et al., 2009; de Klein et al., 2010; Malcolm et al., 2014; Selbie et al., 2015).

The biological exudation of N process inhibitors is also an emerging option for reducing N_2O emissions from grazed grasslands. Compounds could also potentially enter the soil via root death, or, if present in shoot material it could potentially be consumed by livestock and if protected from degradation in the rumen, excreted in the urine. Several plants have been identified to exude nitrification inhibiting compounds including the tropical grass *Brachiaria* spp. and Sorghum, however, the capacity varies between species and further establishment of the effects under field conditions is warranted (Subbarao et al., 2007; Subbarao et al., 2009; Zeng et al., 2015). Recent studies have also utilised biochar plant-extract complexes, where biological nitrification inhibitors are adsorbed onto the biochar, prolonging their effective period in the soil (Muñoz et al., 2014; Reyes-Escobar et al., 2015). The potential for biological denitrification inhibition to occur via plant secondary metabolites has been shown in Bardon et al. (2014), yet this is still an understudied area. There is also current interest in understanding the physiological and genetic plant traits which regulate N_2O emissions, in order to potentially improve agronomic performance alongside environmental sustainability (Thomson et al., 2012).

8.6.2 Soil-based mitigation strategies

In addition to the application of synthetic nitrification inhibitors, inhibiting or promoting other N cycle processes could also reduce N_2O emissions. The nitrification inhibitor DCD has been shown to be more effective in reducing N_2O emissions, NH_3 volatilisation and pasture N uptake from cow urine when applied in combination with a urease inhibitor e.g. N-(n-butyl) thiophosphoric triamide (nBTPT), than when applied alone (Zaman et al., 2009). Although urease inhibitors are not particularly effective at reducing N_2O emissions when used solely, the combined use with nitrification inhibitors is a sensible approach, as the use of nitrification inhibitors alone can increase ammonia volatilisation, as the N remains in the soil as ammonium for longer (Davies and Williams, 1995).

Saggar et al. (2013) describes denitrification as both an environmental threat and an opportunity. The loss of N as N_2O via denitrification could be prevented, or on the other hand, promotion of complete denitrification to N_2 via nitrous oxide reductase is another approach to reducing N_2O (Thomson et al., 2012). Factors which influence the product ratio of N_2O/N_2 includes soil pH, which has led to the suggestion of liming as an N_2O mitigation strategy (Clough et al., 2003; Zaman et al., 2007; Bakken et al., 2012; McMillan et al., 2015). The use of lime has been studied for potential effects on encouraging complete denitrification to N_2, and the use of the soil amendment zeolite has also been considered for reducing the NH_4^+ pool available for nitrification (Zaman et al., 2007; Zaman et al., 2008; Zaman and Nguyen, 2010; McMillan et al., 2015). Zeolite can absorb NH_4^+, as it is a negatively charged natural alumino-silicate (Zaman et al., 2007), which may reduce the available supply of NH_4^+ for nitrification and the amount of available downstream NO_3^- for denitrification. Zeolite has been found to reduce N_2O emissions from urine-influenced soils, and liming has been shown to increase the amount of N_2, as opposed to N_2O from urine-influenced soils (Zaman et al., 2007; Zaman et al., 2010).

Biochar is an additional soil amendment, which, alongside increasing C sequestration in grassland may also have benefits for N cycling in grazed pastures (Taghizadeh-Toosi et al., 2011; Taghizadeh-Toosi et al., 2012). Biochar has been increasingly recognised for its interaction with the N cycle, where the NH_3 and NH_4^+ can absorb into the porous structure (Clough et al., 2009a). Recently, biochar has also been found to reduce the N_2O/N_2 product ratio, facilitating the last step of denitrification, possibly by facilitating the transfer of electron to denitrifiers (Cayuela et al., 2013). In urine-influenced soil the effect of biochar on denitrification appears to be mixed, where some trials have shown a reduction (Taghizadeh-Toosi et al., 2011) and others have shown no change in nitrous oxide emissions (Clough et al., 2009a; Anderson et al., 2014).

Copper has been shown to down regulate the nitrous oxide reductase gene, *NosZ* (Richardson et al., 2009; Sullivan et al., 2013), therefore, when copper is in limited supply the fraction of N released as N_2O from soils may increase. Ensuring copper in soil is non-limiting is a potential strategy to avoid this, via the use of copper-based fertilizers, although to our knowledge, this has not been assessed in any field trials. As denitrification is inhibited by oxygen, techniques which promote soil aeration and reduce soil compaction could be important to consider (Eckard et al., 2010). Moving the location of water and feed containers in the field would prevent several urine patches being deposited to the same area, which can exacerbate N losses (Shepherd and Chambers, 2007; Matthews et al., 2010). Other areas such as sheep tracks and gateways could potentially have greater N_2O emissions, which could be targeted within N_2O emission reduction strategies (Matthews et al., 2010).

8.6.3 Animal-based mitigation strategies

Animal-based strategies to reduce N_2O emissions would involve the selective breeding of ruminants for ‘environmentally friendly’ traits. While there are no such breeding

interventions taking place to our knowledge, the suggestion of breeding livestock which walk while they urinate or urinate more frequently have been made (de Klein and Eckard, 2008; de Klein et al., 2010), in order to improve the spread of urine N across the pasture. Additional improvements may also be made by improving the N conversion efficiency in the rumen of livestock i.e. the amount of N converted to meat or milk (de Klein et al., 2010).

8.6.4 Management strategies

Several management strategies could be employed for tightening the N cycle in grazed pastures. The rate, source, timing and method of fertilizer applications should be carefully considered to ensure it matches the peak N demand of plants and it should not applied under conditions of heavy rainfall (Eckard et al., 2010). The interactive effect of fertilizer application and time of urine deposition has also been shown to influence N_2O emissions and NO_3^- leaching (Anger et al., 2003; Buckthought et al., 2011). Grazing could be restricted for a period of time following fertilizer application as a potential solution to avoid any fertilizer-urine interaction effects. Restricting grazing at other risk periods e.g. winter periods where rainfall is high and compaction by hooves is more likely (de Klein et al., 2006), has also been suggested as an N_2O mitigation strategy. Effluent from livestock can then be collected and re-applied when N demand and soil conditions are appropriate, which can also minimise N losses. While this is an option for certain cattle systems, sheep grazing is often extensive, where animals graze all year round. Further research is required to determine mitigation strategies specifically for extensive systems.

The avoidance of camping areas through grazing management may reduce N_2O emissions, such as through grazing management e.g. rotational grazing, as opposed to continual grazing (Petersen et al., 2004; Dahlin et al., 2005). On the other hand, knowledge of camping areas could be viewed as an opportunity for the use of targeted mitigation strategies e.g.

nitrification inhibitors. Further studies are required on rotational grazing systems to determine how differences in management strategies effect N_2O emissions. Generally, increasing stocking rates will increase N_2O emissions, however, rotational grazing can cause less or more N_2O emissions depending on management, climatic and regional factors (Saggar et al., 2007). Silvopastoral systems may increase shading/shelter areas for livestock, where tree roots may help acquire N leached beyond the pasture root zone, which could be emitted as N_2O indirectly (Schoeneberger et al., 2012).

Balancing the protein-to-energy ratio of ruminant diets can reduce the amount of urinary N excreted. As discussed previously, this could be achieved through the use of high-sugar fodder varieties; alternatively the provision of high-energy supplements can be provided where animals are grazing high protein grass (Eckard et al., 2010). The inclusion of salt, or other diuretic compounds may reduce the N load per urination by increasing total urinary volume and increasing the spread of urine across the pasture (Ledgard et al., 2007; Costall and Betteridge, 2010; Pacheco et al., 2010; Liu and Zhou, 2014).

8.6.5 Demand-led strategies

The simplest strategy to reducing N_2O emissions from urine patches would be to decrease livestock numbers, and hence the total amount of excreta returned to land (Luo et al., 2010; Ussiri and Lal, 2012). This could be achieved through a reduction in meat protein consumption *per capita,* or even a complete switch to plant-based protein consumption, which under simulated modelled scenarios have been shown to cause large reductions in agricultural non-CO_2 greenhouse gases (Stehfest et al., 2009; Popp et al., 2010; Westhoek et al., 2014). Nevertheless, changes in the supply and demand for animal products and the change in land-use associated with any reduction in animal product consumption are highly uncertain (Westhoek et al., 2014; Decock et al., 2015). In addition, the effects on livestock producers and

downstream processing and supply chains, which provide income to rural economies, are likely to be negative and have to be considered (Westhoek et al., 2014). Livestock production can also occur on marginal land, which is not suitable for growing crops (Kingston-Smith et al., 2010). Encouraging a reduction in animal product consumption, alongside reductions in food wastage and better transport and supply chains, is a sensible strategy to reduce agricultural GHG emissions, nevertheless, it is considered unlikely that widespread vegan/vegetarianism would be adopted and with increasing population, global projections point in the opposite direction (Smith, 2010; Thornton et al., 2010). Direct mitigation strategies at the farm-scale will inevitably have an important role to play in emission reductions from the livestock sector in the short term.

Despite a wide range of potential mitigation strategies for N_2O, implementation of techniques are lagging, and the development of N_2O specific policies may be required (Schils et al., 2013). Nitrification inhibitors, fertilizer management and managing animal diets have been considered to show the best potential for N_2O emission reductions in the short term (de Klein and Eckard, 2008). Whilst some of the techniques described here need further research and development, the solutions adopted by farmers will more than likely depend on the practicality and economics of individual farms (Luo et al., 2010). Strategies to reduce N_2O emissions from urine patches could be incorporated into agri-environmental schemes, in order to encourage adoption. Farmers may need assistance in an advisory capacity, to manage N efficiently and to ensure the avoidance of pollution swapping. Several of the suggested mitigation strategies are more suited to intensive livestock systems, and further novel strategies may be required under more extensive systems (Eckard et al., 2010).

8.7 How should future research be prioritised, in order to achieve enhanced productivity and sustainability in grazed grasslands?

Achieving enhanced productivity and sustainability in grazed grassland is a complex challenge, which will require a multidisciplinary approach to resolve. In order to understand the spatial and temporal variability of soil processes and N transformations at the scale of the urine patch, advances will need to be made in order to monitor urine patches in as non-destructive a manner as possible. The use of planar optodes to measure changes in soil pH, O_2, CO_2 and NH_4^+ have been described for use in the rhizosphere (Blossfeld et al., 2011; Blossfeld, 2013), where such technology may also be useful for studying urine-influenced soil, although the technique is confined to two dimensions. The use of N_2O microelectrodes (Elberling et al., 2010) and the development of *in-situ* electrodes to monitor soil mineral N (Shaw et al., 2013), could provide a method of studying the urine patches without causing excessive soil disturbance. Fluorescence *in situ* hybridization (FISH) based approaches have been reported for use in identifying microbial hotspots and microbial groups within hotspots in either 2D or 3D surfaces (e.g. soil root interface; Musat et al., 2008; Schmidt et al., 2012; Schmidt and Eickhorst, 2013; Schmidt and Eickhorst, 2014; Kuzyakov and Blagodatskaya, 2015). Knowledge of wider N cycling in the urine patch is lacking, where several process rates need further study, such as determining nitrification, denitrification and mineralization in differing zones of the urine patch. There is limited research regarding the influence of urine on DOC and DON solubilisation, and whether the increase in DOC fuels denitrification. Modelling N process rates in the urine patch using ^{15}N tracing models would provide useful insight into the fate of urinary N (Müller et al., 2004; Müller et al., 2007; McGeough et al., 2016). Recently, N_2O has also been shown to be produced by ammonia-oxidising bacteria present on the leaves of pasture plants influenced by urine, where further investigations should be conducted to quantify the magnitude of this N_2O source (Bowatte et al., 2015).

Understanding the source partitioning (major soil process; nitrification or denitrification) of N_2O release from soil will be fundamental for improving N_2O mitigation strategies (Köster et al., 2013), as some techniques may prevent one microbial N_2O pathway but promote another. It may also improve understanding of the environmental regulators of N_2O production pathways, aside from nitrification and denitrification. Advances in technology able to continuously monitor the isotopomers and site preference of N_2O in the field, promise a greater understanding of the source partitioning of N_2O production, where this technique is currently known to distinguish between N_2O produced via hydroxylamine reduction, nitrate reduction, and between bacterial and fungal denitrification (Thomson et al., 2012). The 'omics' (genomics, proteomics, metabolomics) era is also another important research area, which could advance understanding of N cycling and losses in the urine patch. Additionally, omics of urine itself may provide better knowledge of urinary compounds, some of which are known to influence N_2O production and emission from soil e.g. hippuric acid and benzoic acid (Kool et al., 2006; Bertram et al., 2009; Clough et al., 2009b). There is a general need for more research relating the microbial composition and functioning in urine-influenced soils, in relation to N_2O emissions. Process-based models will continually need to be developed and updated to incorporate advances in our knowledge of N_2O production in soils.

In terms of mitigating N losses from the urine patch efficiently, targeting the areas of the pasture in space and time is another challenge which needs to be met. A few novel technologies have been made and are continuing to be developed for cattle-based systems, including the use of a tail mounted nitrification inhibitor dispensing device, which dispenses a given amount of inhibitor following urination (Bates and Quin, 2013). The use of robotised vehicles have also been developed (Bates et al., 2015), sensing where the urine patches are located via fluorescence, temperature or chlorophyll measurements. Nevertheless, the time taken for the pasture to respond to a urine application can be long after the N loss risk period,

and temperature can dissipate too quickly for these strategies to be useful (Bates and Quin, 2013; Bates et al., 2015). This has led to the more recent development of a towable or automated technique, of measuring the electrical conductivity and moisture content of the pasture soil, which resulted in a good detection of urine patches (Bates et al., 2015). This method of sensing the urine patch has been described for use with a combination of a urease inhibitor, gibberellic acid and a dissolved organic C supply (a fulvic acid polymer), in order to delay urea hydrolysis, promote plant growth in the urine patch and reduce N losses, where preliminary trials showed a comparative efficacy to DCD (Bates et al., 2015). Further advances in such technologies will be important for providing practical and cost-effective measures to reduce N losses at the farm scale.

Care should be taken when selecting mitigation strategies, to ensure that 'pollution swapping' does not occur. Some strategies which reduce N_2O emissions may exacerbate losses of other N forms, or other greenhouse gases e.g. housing livestock would result in manure piles which generate ammonia, and would require bought in concentrates, which is more carbon intensive. When considering mitigation strategies, a whole life cycle approach needs to be considered, in order to consider the potential trade-offs which may result due to implementation of such measures.

8.8 Conclusions

The N concentration and size of sheep urine patches have been shown to influence N_2O emissions, therefore, further data is required on urine volume, frequency and N concentrations under a range of management techniques and climatic variables. Environmental parameters exert a strong influence on N_2O emissions from urine patches, where measured N_2O emissions from sheep urine patches under summer conditions were lower than the default IPCC Tier 1 approach to calculating N losses from sheep excreta. A move to Tier 2 and 3 approaches will

be fundamental to better constrain N_2O emission estimates from grasslands, based on variability in soil type, hill slopes, climate and management related factors.

When utilising chambers to calculate N_2O emission factors from sheep urine patches, sufficient room should be allowed for the urine patch diffusional area (and potentially the pasture response area) in order to reflect emissions as closely as possible. If using a manual sampling campaign sampling should be conducted as close to daily as possible, an assessment of the diurnal variability of N_2O emissions is recommended and chamber number should be maximized under both manual and automated campaigns, in order to adequately capture spatial variation in N_2O emissions.

The capacity for plants to directly acquire DCD and translocate this compound to the shoots, as demonstrated in this thesis, represents a potential vector for the contamination of livestock products when using inhibitor, where the use of synthetic nitrification may attract livestock product consumer scepticism. This combined with the apparent lack of effectiveness of DMPP in reducing N_2O emissions from sheep urine patches, suggests the use of nitrification inhibitors alone will not be sufficient to entirely reduce N_2O emissions from urine patches. The efficacy of nitrification inhibitors does vary widely, yet, they represent a mitigation strategy which could be implemented in the short term.

Achieving enhanced sustainability and productivity in livestock systems is a challenging and complex problem, which will require expertise and collaboration across conventional scientific disciplines, policy-makers and farmers. There are, however, several mitigation strategies available, being developed, or some which require much more research before being practicable. Advances in technologies to measure and mitigate N_2O emissions will greatly enhance our knowledge of N cycling in the urine patch environment in the near future.

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Appendix 1

Supplementary material for Chapter 3

Figure 1 Eutric Cambisol mesocosms maintained at 50% and 70% WFPS.

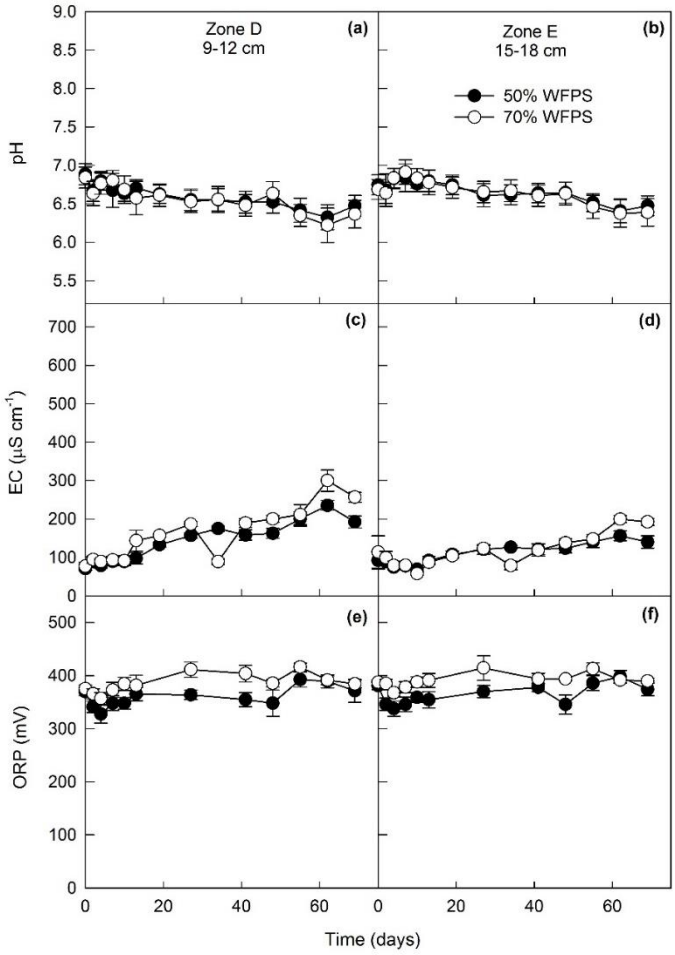


Figure 2 Changes in soil pH (panels a and b), electrical conductivity (EC; panels c and d) and oxidation reduction potential (ORP; panels e and f) following sheep urine application to a Eutric Cambisol, maintained at either 50% or 70% water-filled pore space (WFPS), and sampled at 9-12 cm (panels a, c and e) and 15-18 cm (panels b, d and f) away from the direct area of application. Symbols represent means ± SEM (n = 4). Figure legend applies to all panels and text on the top row of panels applies to each respective column.

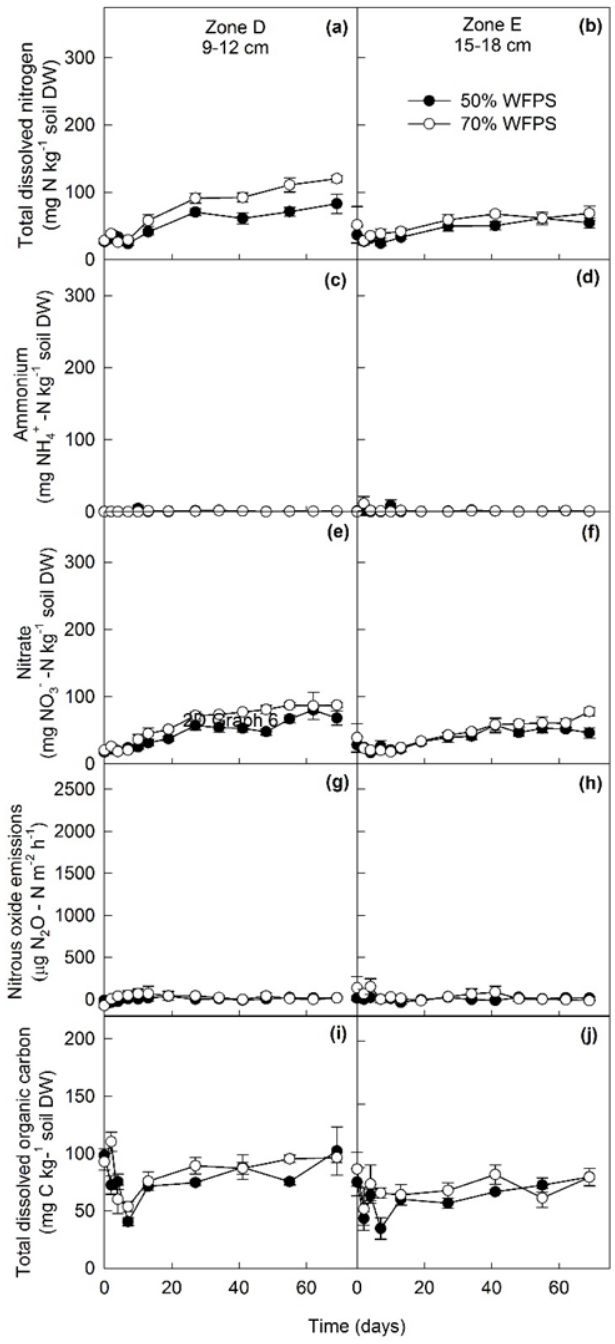
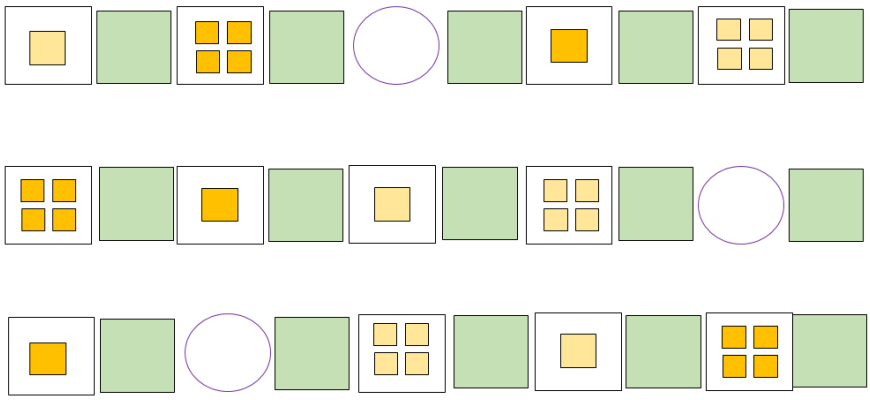


Figure 3 Extractable total dissolved nitrogen (panels a and b), ammonium (panels c and d), nitrate (panels e and f), nitrous oxide emissions (panels g and h) and total dissolved organic carbon (panels i and j) following sheep urine application to a Eutric Cambisol, maintained at either 50% or 70% water-filled pore space (WFPS), and sampled at 9-12 cm (panels a, c, e, g and i) and 15-18 cm (panels b, d, f, h and j) away from the direct area of application. Symbols represent means ± SEM (n = 4). Figure legend applies to all panels and text on the top row of panels applies to each respective column.

Appendix 2

Supplementary material for Chapter 4



Key:

Automated chamber

Manual chamber

Duplicated soil sampling plots

Low N concentration large urine patch (4 g N l^{-1}; 1 g N total; 200 kg N ha^{-1})

High N concentration large urine patch (16 g N l^{-1}; 4 g N total; 800 kg N ha^{-1}; 500 cm^2)

Four low N concentration small urine patches (4 g N l^{-1}; 4 × 0.25 g of N total, applied in 4 × 62.5 ml, covering 125 cm^2 each; 200 kg N ha^{-1})

Four high N concentration small urine patches (16 g N l^{-1}; 4 × 1 g of N total, applied in 4 × 62.5 ml, covering 125 cm^2 each; 800 kg N ha^{-1})

Figure 1 Plot layout for the field trial conducted in Chapter 4.

Appendix 3

Details of an attempted study using lux marked *Nitrosomonas europeae*

This study utilised a lux marked strain of the nitrifying bacterium *Nitrosomonas europeae* ATCC19718 (pHLUX20). Unfortunately, some problems occurred during experimentation, and the long culture time of this microorganism made it difficult to troubleshoot problems with the bio assay. Due to time constraints, it was decided to discontinue the study. A summary of the main objectives and some photographs of culturing this microorganism are shown below (Fig 1).

The objectives of this experiment were to test whether any compounds commonly excreted in the urine of ruminants, are able to inhibit nitrification. If any compounds within urine do inhibit nitrification, then it may be possible to increase excreted inhibitor concentrations through diet. Excretion of the inhibitor would then theoretically be deposited in the exact location it is required – which may save money/labour costs in comparison to e.g. blanket applications of nitrification inhibitors.

The following objectives were planned:

1) Monitor growth of *Nitrosomonas* culture by measuring OD_{600}
2) Confirm that inhibition of fluorescence corresponds to inhibition of nitrification (i.e. % nitrite production in assay)
3) Screen the following urine compounds for their inhibitory effect on nitrification: allantoin, uric acid, creatinine, creatine, hippuric acid, benzoic acid and compare with chemical nitrification inhibitors e.g. DCD, DMPP and the standard Allylthiourea

4) Narrow down compounds which have an inhibitory effect, and then test the mode of action of inhibition i.e. test whether the ammonium oxidation (AMO) or the hydroxylamine pathway (HAO) pathway is interfered with.
5) Test inhibition of nitrification in soils in the lab, determine the inhibitory potential of a concentration range of the nitrification inhibitors, a concentration range within artificial urine and finally by spiking urine with increased amounts of inhibitor.
6) Test the potential for N_2O emission reduction in soils – pot trial.

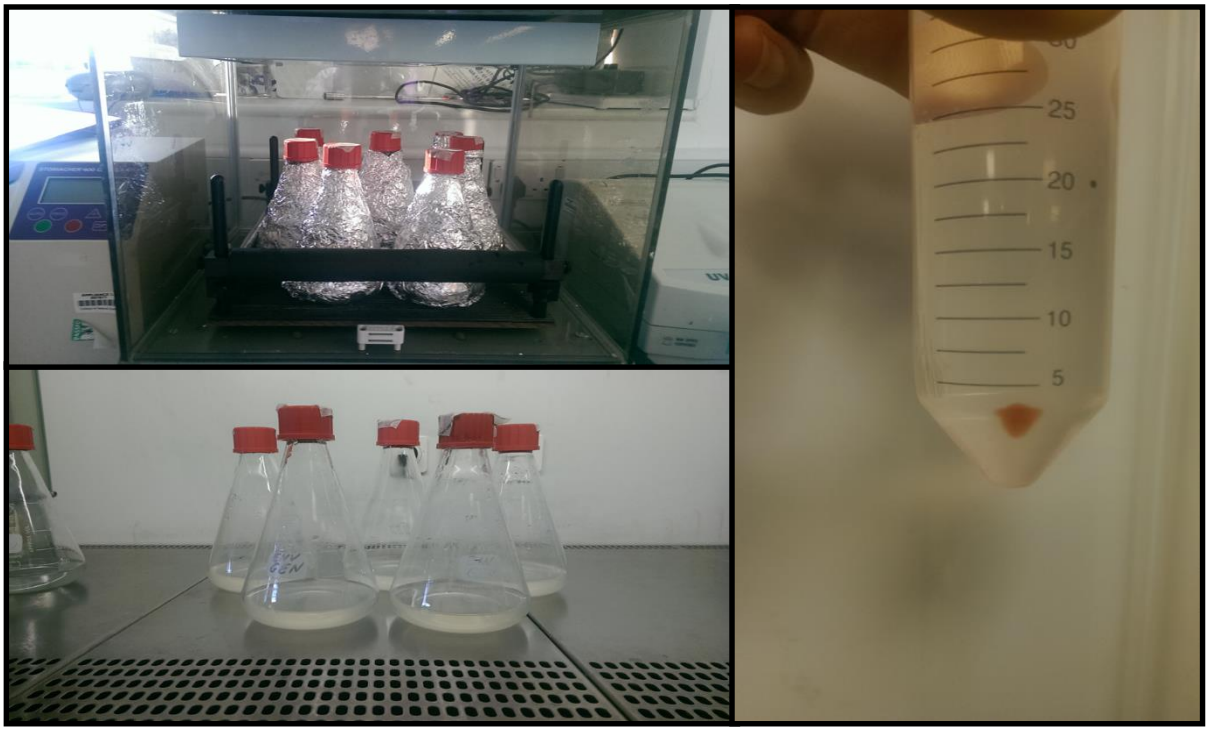


Figure 1 Culture of a lux marked strain of the nitrifying bacterium *Nitrosomonas europeae* ATCC19718 (pHLUX20).